Preparation and Characterization of Regenerated Cellulose Microspheres and the Adsorption of Pectinase

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Porous cellulose beads were prepared through a simple, facile, and inexpensive method. The resultant microspheres exhibited good spherical shape with a diameter of 1 to 2 mm. Their morphology, pore structure, and physical properties were characterized by scanning electron microscopy, X-ray diffraction, and nitrogen adsorption. The regenerated cellulose was shown by scanning electron microscopy images to have a three-dimensional porous structure, which led to a BET surface area as large as 108 m^2/g. These qualities make the beads potentially useful as adsorbents or carriers. The beads remained in the cellulose I structure. Finally, the cellulose beads were tested for the adsorption of pectinase; adsorption was a favorable spontaneous process. Moreover, adsorption was in agreement with the Langmuir isotherm with a capacity of 7.40 mg/g, signifying that pectinase adsorption was a monolayer sorption. Adsorption followed an intraparticle diffusion kinetic model, indicating that intraparticle diffusion was the rate-controlling mechanism. This information will aid in the potential utilization of regenerated cellulose microspheres as supports for pectinase.

Keywords: Cellulose; Enzymes; Pectinase; Adsorption; Isotherm; Kinetics

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INTRODUCTION

Because environmental pollution threatens our planet, finding novel, environmentally friendly processes and materials is crucial. Materials from renewable resources, especially biodegradable polymers, are key factors, and cellulose is an excellent example. It is the most abundant, renewable, and biodegradable polymer on Earth. Moreover, cellulosic materials are generally hydrophilic, insoluble in water, inert, nontoxic, and inexpensive (Klemm *et al*. 2005).

Natural cellulose is usually found in crystalline fibrils, which limit its potential applications but drive interest in regenerated cellulose (Sannino *et al.* 2009). Regenerated cellulose microspheres have large specific surface areas, porosities, good adsorbing capacities, and high mechanical strengths. The spherical shape guarantees excellent flow properties in column systems (Gericke *et al.* 2013), and they have been used as chromatographic supports, adsorbents for heavy metals, superabsorbents, and supports for solid phase synthesis (De Luca *et al.* 2003; O'Connell *et al.* 2008; Luo and Zhang 2010a; Demitri *et al.* 2013; Gonte *et al.* 2013; Zhang *et al.* 2013). Furthermore, the excellent biocompatibility of cellulose has prompted its use as a carrier for proteins, enzymes, cells, and pharmaceuticals (Weber *et al.* 2005; Esaki *et al.* 2009; Luo *et al.* 2009; Volkert *et al.* 2009). A number of enzymes including glucose oxidase, penicillin G acylase, invertase,

laccase, β-galactosidase, α-chymotrypsin, *etc*., have been successfully immobilized on regenerated cellulose spheres (Robinson *et al.* 1973; Roy and Gupta 2003; Turner *et al.* 2005; Bryjak *et al.* 2008; Luo and Zhang 2010b; Liu *et al.* 2012). The beads are also suitable carriers for pectinase. Pectinase is immobilized *via* adsorption, and it exhibits good catalytic performance and stability in a previous study (Wu *et al.* 2014). Thus, it is worthwhile to further explore the properties of cellulose beads and the adsorption behavior of pectinase on these beads.

In this work, porous spherical cellulose beads were prepared from microcrystalline cellulose by a simple and economical method. The properties of the regenerated cellulose beads were characterized. The adsorption of pectinase onto the cellulose beads was investigated, and the equilibrium isotherm and adsorption kinetics models were evaluated to analyze the adsorption process. This report elucidates the potential utilization of regenerated cellulose microspheres as enzyme supports or adsorbents in biotechnology and biochemistry.

EXPERIMENTAL

Materials

Microcrystalline cellulose was purchased from Sinopharm (Shanghai, China). Liquid Pectinase Novozym 51019 was supplied by Novozymes (Copenhagen, Denmark). Analytical grade LiCl, N,N-dimethylacetamide (DMAC) and all other chemicals were purchased from Tianjin Fuchen Chemical Reagents Factory (Tianjin, China).

Preparation of Regenerated Cellulose Beads

A 3 wt% cellulose solution was prepared as described with some modifications (Araki *et al.* 2006). Microcrystalline cellulose (3.0 g) was dispersed in 89.0 g of DMAC and heated at 150 °C for 30 min. After the mixture was cooled to 100 °C, 8.0 g of LiCl was added to the mixture and stirred for 30 min. The mixture was cooled to room temperature and stirred overnight. The solution was added dropwise to ethanol to obtain regenerated cellulose gel. The gels were washed thoroughly with deionized water. An $AgNO_3$ test detected Cl^- in the washings to confirm that the beads were free from LiCl. The absence of DMAC was confirmed by elemental analysis of the dried samples using a Vario EL cube (Elementar, Frankfurt, Germany). The diameter of the resultant wet beads was approximately 1 to 2 mm. All the samples were freeze-dried before use.

Characterization of the Regenerated Cellulose Beads

Scanning electron microscopy (SEM) was used to observe the morphology of the microspheres. The samples were freeze-dried, sputtered with gold, and examined using a Zeiss EVO 18 device (Oberkochen, Germany). X-ray diffraction (XRD) patterns of the microcrystalline cellulose and regenerated cellulose beads were recorded on an XRD instrument (D8 ADVANCE, Bruker, Karlsruhe, Germany). The crystalline peaks were fitted using a Lorentz function in an OriginPro 8.6 software (OriginLab, MA, USA). The spacing of the crystal planes was calculated according to Bragg equation,

$$d = \lambda/(2 \sin\theta) \quad (1)$$

where d is the spacing, λ is the wavelength (0.15418 nm), and θ is the diffraction angle. The Z-value was calculated by $Z = 1693\ d_1 - 902\ d_2 - 549$ (d_1 is the spacing of peak 1 and

d_2 is the spacing of peak 2). The apparent crystal size was evaluated from the Scherrer equation based on 002 spacing,

$$L=K\lambda/(\beta \cos\theta) \quad (2)$$

where L is the crystallite size, K is Scherrer constant of 0.9, and β is the width of the diffraction peak measured at half maximum height.

Nitrogen adsorption measurements were performed with a Micrometrics ASAP 2020 (Atlanta, USA). The sample was degassed at 40 °C. Brunauer-Emmett-Teller (BET) analysis was carried out with a N_2 relative vapor pressure of 0.05 to 0.3 at 77 K.

Adsorption Equilibrium Study

Batch adsorption experiments were performed by immersing 0.0500 g of regenerated cellulose beads in 10 mL of pectinase solution of different concentrations and constantly shaking the mixture in a gas bath at 20 °C for 72 h. The amount of enzyme adsorbed on the microspheres was estimated by a UV absorption method (Walker 2002), where the final protein present in the enzyme solution was subtracted from the initial protein. Protein mass was determined on a balance. The equilibrium data were analyzed according to both Langmuir and Freundlich models to understand the adsorption further. The Langmuir isotherm can be expressed as follows:

$$C_e/q_e = C_e/q_m + 1/(\underline{bq}_m) \quad (3)$$

where q_e (mg/g) is the amount of enzyme adsorbed at equilibrium, C_e (g/L) is the equilibrium solute concentration, q_m (mg/g) is the maximum uptake, and b (L/g) is the Langmuir adsorption constant related to the energy of sorption. Both q_m and b can be determined from the linear plot of C_e/q_e versus C_e.

The Freundlich model assumes that the adsorption amount increases with the increment in the concentration according to Eq. 2,

$$q_e = k_f C_e^{1/n} \quad (4)$$

where k_f is the Freundlich isotherm constant and $1/n$ (dimensionless) is the heterogeneity factor.

The favorable nature of adsorption was expressed in terms of a dimensionless separation factor, R_L ($R_L = 0$ for the irreversible case, $0 < R_L < 1$ for favorable equilibrium, $R_L = 1$ for the linear case, and $R_L > 1$ for unfavorable equilibrium), as defined by Eq. 3 (Hall *et al.* 1966),

$$R_L = (1 + b\ C_0)^{-1} \quad (5)$$

where C_0 (g/L) is the initial concentration of pectinase in solution.

The change in Gibbs apparent free energy (ΔG) was calculated using Eq. 4:

$$\Delta G = -RT \ln K_L \quad (6)$$

The Langmuir equilibrium constant K_L (L/mol) was obtained by multiplying the value of b with the molar weight of the pectinase.

Adsorption Kinetics

A mixture of regenerated cellulose microspheres (0.0500 g) and 10 mL of pectinase solution (0.177 g/L) was shaken using a shaker at 20 °C. The protein concentration in the solution was measured at intervals, and the amount of adsorbed enzyme was calculated by

means of a mass balance as described before.

A linear form of the pseudo-first-order model was described by Lagergren (1898),

$$\ln (q_e - q) = \ln q_e - k_1 t \quad (7)$$

where q_e and q are the amounts of enzyme (mg/g) adsorbed at equilibrium and at time t, respectively, and k_1 (min^{-1}) is the rate constant of first-order adsorption. The second-order kinetic model is expressed in Eq. 6,

$$t/q = 1/v_0 + t/q_e \quad (8)$$

where $v_0 = k_2 q_e^2$, the initial sorption rate, and k_2 (g/mg·min) is the rate constant of pseudo-second-order adsorption (Ho and McKay 1999; Reddad *et al.* 2002).

RESULTS AND DISCUSSION

Structure Characterization

The diameter of the resultant wet beads was approximately 1 to 2 mm. The surface structure of regenerated cellulose microspheres was observed by SEM (Fig. 1). The microspheres exhibited good spherical shapes and porous structures. The microspheres were formed by hydrogen bonding and possibly chain entanglement because no crosslinking agent was used (Chang and Zhang 2011). The pores were formed by ethanol-induced phase separation, during which the solvent-rich regions were transformed into pores (Bognitzki *et al.* 2001). There were small and large holes on the surface (Figs. 1b and c, respectively), probably because the solvent transformation rate varied as the cellulose was dripped into the ethanol. Also, the large pores penetrated the bead interior so that a three-dimensional network was formed (Fig. 1d). Cross-sections of microspheres showed that the bead core was far less dense than the wall (Figs. 1e, f). The pores in the core were arranged in a radial pattern, and homogeneous pores were observed in the wall, which suggested a three-dimensional network. The regenerated cellulose beads possessed a large specific surface area due to this three-dimensional network. As evaluated by BET analysis, the specific surface area of the microspheres was 108 m^2/g, which was much higher than reported values (Luo and Zhang 2010b). These features indicate that the regenerated cellulose microspheres are good carriers or adsorbents.

XRD Analysis

XRD patterns of the original microcrystalline cellulose and regenerated cellulose beads were shown in Fig. 2. A Lorentz function was used to examine the X-ray diffraction peak intensity and distribution. The characteristic cellulose I crystalline peaks were present at 101, 10$\bar{1}$, 021, 002, and 040 (Fig. 2, peaks 1 through 5, respectively); peak 6 represented the amorphous phase (Hult *et al.* 2003; Park *et al.* 2010). Regenerated cellulose reportedly has cellulose II structure (Cai *et al.* 2008; Liu *et al.* 2012), but in this study, the regenerated cellulose beads exhibited cellulose I crystalline peaks (Fig. 2b). The cellulose solution was observed under polarizing microscope and it is shown that a very small number of cellulose fragments existed in the solution. Thus, it is likely that an all-cellulose composite structure formed in the regenerated microspheres. LiCl/DMAC may peel away of layers from the original crystalline cellulose. After the solvent is removed, these layers form a paracrystalline phase that is distinct from typical amorphous cellulose and closer in structure to cellulose I (Benoît *et al.* 2007; Huber *et al.* 2012).

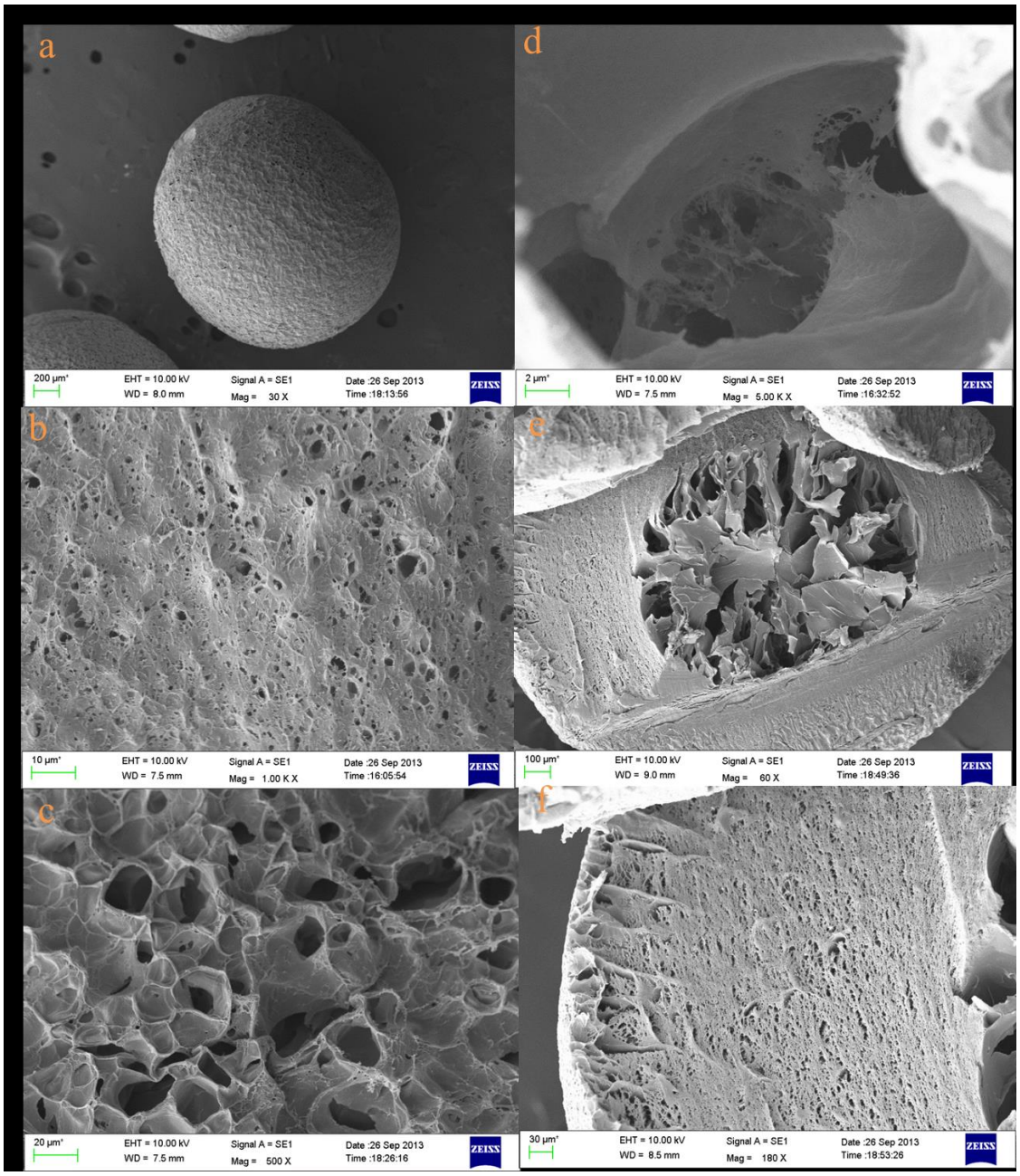

Fig. 1. SEM of the surface morphology (a), surface structure (b, c, d), and cross-section structure (e, f) of regenerated cellulose beads

The crystallinity of the samples was estimated based on the ratio of the area of all crystalline peaks to the total area. The crystallinity of the initial microcrystalline cellulose and the regenerated cellulose was 95.0% and 57.7%, respectively. The decreasing crystallinity resulted from the cleavage of intra- and intermolecular hydrogen bonds in cellulose during the dissolution and gelation processes, which rendered the sample more disordered. The regeneration step could prevent dissolved cellulose from recombining into the original crystalline structure (Li *et al.* 2009). In addition, the lower crystallinity meant the microspheres had proportionally more of the amorphous regions, probably due to the formation of porous structures (Luo and Zhang 2010a).

The spacing of the crystal planes was calculated (Table 1). There were minor changes in the spacing of crystallographic planes between the original and the regenerated cellulose. The Z-value can be used to discriminate between I_α (triclinic) and I_β (monoclinic) crystalline phases. The Z-values of the raw material and the beads were both negative, suggesting that the regenerated cellulose microspheres remained I_β dominant (Wada *et al.* 2001). Moreover, the apparent crystal size was evaluated from the Scherrer equation for the initial microcrystalline cellulose and the regenerated cellulose; the latter sample had a slightly lower crystallite size based on 002 spacing.

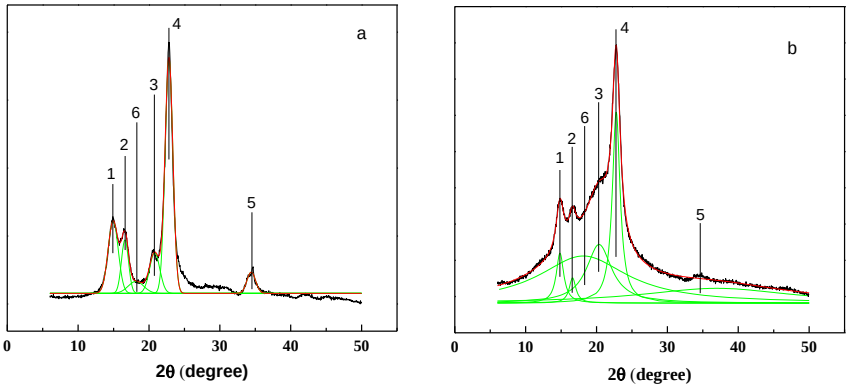

Fig. 2. XRD patterns of microcrystalline cellulose (a) and regenerated cellulose microspheres (b)

Table 1. Properties of Microcrystalline Cellulose and Regenerated Cellulose Beads

Sample	Lattice Spacing (nm)					Z-Value	Crystallite Size (nm)	Crystallinity (%)
	d_1	d_2	d_3	d_4	d_5			
Microcrystalline Cellulose	0.299	0.269	0.217	0.199	0.136	-286	6.66	95.0
Regenerated Cellulose beads	0.301	0.269	0.221	0.199	0.128	-283	6.49	57.7

Adsorption Isotherm

The equilibrium isotherm describes interactive behaviors between solutes and adsorbents. Equilibrium sorption studies were used to investigate the adsorption of pectinase by regenerated cellulose microspheres (Fig. 3). The equilibrium adsorption, q_e, increased rapidly with increasing enzyme concentrations at low concentrations.

The equilibrium data were analyzed according to the Langmuir and Freundlich models (Table 2). The Langmuir model was more suitable for simulating the adsorption isotherm of pectinase in the regenerated cellulose microspheres; the fitting result by Langmuir model is shown in Fig. 3b. This result was similar to other reports (Fargues *et al.* 1998; Bautista *et al.* 2000). Langmuir isotherm model assumes that the adsorption occurs on a structurally homogeneous surface where all adsorption sites have equal energies and enthalpies of adsorption and only monolayer adsorption occurs in the process. Therefore, the result implies that the enzyme developed a homogeneous monolayer on the beads and that the interaction between the adsorbed molecules on the support was marginal (Fatehi *et al.* 2013). Moreover, q_m calculated based on Langmuir model was quite close to the experimental data.

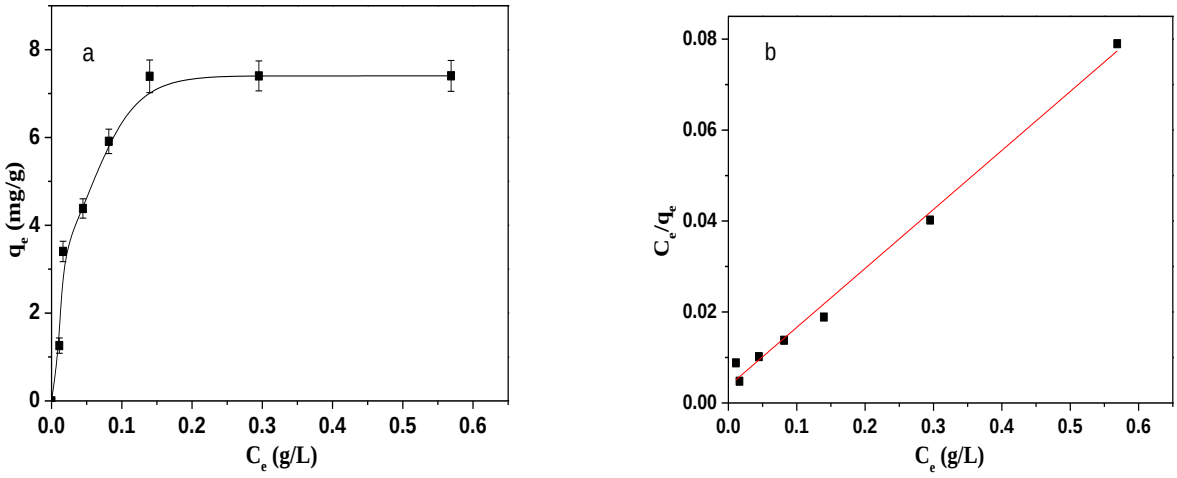

Fig. 3. Adsorption isotherm of enzyme uptake on regenerated cellulose beads at 20 °C.

Furthermore, the value of R_L was calculated to be smaller than 1 at various initial concentrations, thus indicating the adsorption of pectinase was a favorable process.

The molar weight of the pectinase was about 20 to 40 kDa (Yadav *et al.* 2009), and the value of b turned out to be 35.44. Thus, ΔG was negative, which confirmed the feasibility and spontaneous nature of adsorption (Gupta *et al.* 2004).

Table 2. Isotherms Parameters of Pectinase Adsorption onto Regenerated Cellulose Microspheres

$q_{e(exp)}$ *	Langmuir model			Freundlich model		
(mg/g)	*b* (L/g)	q_m(mg/g)	R^2	k_f	*n*	R^2
7.40	35.44	7.71	0.9917	12.27	2.62	0.6927
* Experimental data						

Adsorption Kinetics

Analysis of adsorption kinetics showed that enzyme uptake was rapid during the initial phase of adsorption and slowed until equilibrium was attained (Fig. 4a). As proposed by Sarkar and Chattoraj (1993), the adsorption of biopolymers proceeded in three major steps. Firstly, the biopolymers diffused quickly from bulk to the subphase in contact with the solid surface. Afterward, protein molecules in the subphase formed complexes with active spots on the solid surface, which might have influenced the tertiary structure of the protein. Finally, polypeptide segments not contacting the surface may orient.

There are numerous kinetic models describing the adsorption of protein on solid surfaces (Rabe *et al.* 2011). Here, pseudo-first-order and pseudo-second-order models were tested for their applicability to pectinase adsorption on regenerated cellulose microspheres (Table 3). Based on the coefficients of determination, a pseudo-second-order model fit the experimental data much better (Fig. 4b). Thus, it often has been claimed in the literature that chemisorption was the rate-limiting step involving valence forces through sharing or exchange of electrons between sorbent and sorbate (Ho 2006). However, it is unlikely that there was covalent bonding or ion exchange between enzyme and cellulose beads. As for adsorption rate, the protein molecules might be transported from the bulk solution into the

solid phase through an intraparticle diffusion process, the rate-limiting step in some adsorption processes. It might be that a pseudo-second order expression just happened to fit pretty well when describing adsorption on porous surfaces because it took a long time for the adsorbate to penetrate the pores. Thus, intraparticle diffusion equation was introduced to explore the possibility of the intraparticle diffusion as the rate-limiting step in the adsorption (Weber and Morris 1963),

$$q = k_{id}\, t^{0.5} + C \quad (9)$$

where k_{id} is the intraparticle diffusion rate constant (mg·min$^{0.5}$/g) and C is the intercept. A plot of q versus $t^{0.5}$ is shown in Fig. 4b. A linear relationship was observed in the initial stage of adsorption, thus confirming the involvement and importance of diffusion-controlled transport mechanism in the initial adsorption process. Thereafter, other factors might be important for adsorption (Li and Bai 2005).

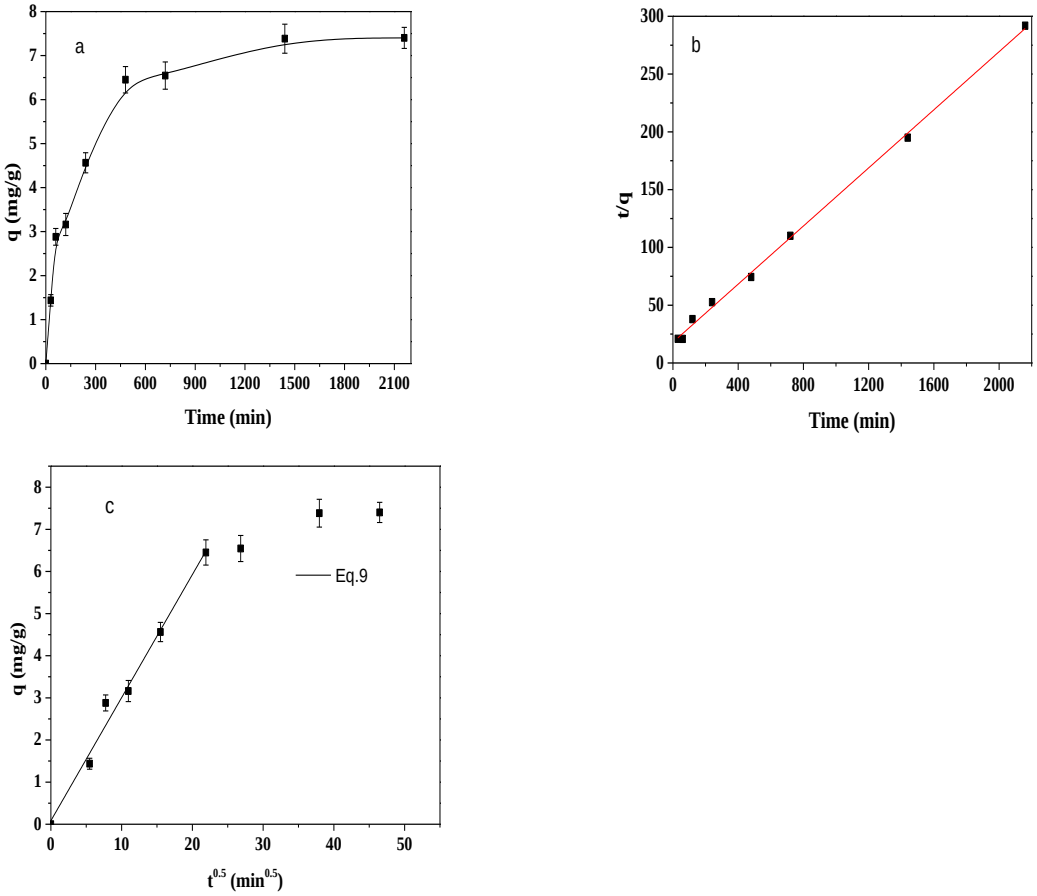

Fig. 4. Adsorption results of enzyme with an initial concentration of 0.177 g/L at 20 °C (a) and the fitting results of the pseudo-second order model (b) and the diffusion-controlled kinetic model (c)

Moreover, the fact that the coefficient of determination for the pseudo-first order model was also high suggests that some weak interactions may also be involved in the adsorption process (Yan *et al.* 2011). Apart from that, hydrogen bonds might form between the sorbent and the sorbate.

Table 3. Kinetics of Pectinase Adsorption on Cellulose Microspheres

Kinetic Model	Calculated Parameters			
Pseudo-First-Order Equation	q_e (mg/g) 7.19	$k_1 \times 10^3$ (min^{-1}) 3.97	R^2 0.970	
Pseudo-Second-Order Equation	q_e (mg/g) 7.96	$k_2 \times 10^4$ (g/(mg·min)) 8.81	R^2 0.998	V_0 (g/(mg·min)) 0.0557
Intraparticle Diffusion	k_{id} ($mg \cdot min^{0.5}/g$) 0.292	C 0.0845	R^2 0.982	

Considering their properties and adsorption capacity, the regenerated cellulose microspheres obtained in this research could be further utilized as a carrier for protein in biotechnology and adsorbent for pollutants in industry. Since cellulose I is the strongest allomorph with a theoretical ultimate tensile strength of about 13 to 17 GPa, the mechanical properties and modification of the microspheres will be studied in the future to consider its application as functional material.

CONCLUSIONS

1. Porous cellulose beads were prepared by dissolving microcrystalline cellulose with the LiCl/DMAC system and re-forming cellulose microspheres in ethanol. The regenerated cellulose microspheres exhibited good spherical shape and high porosity.
2. The regenerated cellulose retained cellulose I structure. The microspheres had lower crystallinity because intra- and intermolecular hydrogen bonds were at least partly broken during dissolution and gelation.
3. The beads adsorbed pectinase in a favorable spontaneous process. Analysis results revealed that the adsorption accorded well with Langmuir isotherm with a capacity of 7.40 mg/g, indicating that the pectinase adsorption was a monolayer sorption and the interaction between the adsorbed molecules on the support was marginal. This was advantageous for the catalytic activity of the enzyme.
4. The adsorption kinetics were described by intraparticle diffusion kinetic model. This meant that pectinase was adsorbed from the bulk solution onto cellulose beads through an intraparticle diffusion process and intraparticle diffusion was the rate-limiting step in the initial adsorption process, though weak interactions were also involved.
5. The results shown that the obtained regenerated cellulose beads could potentially be used as enzyme carriers or adsorbents.

ACKNOWLEDGMENTS

This work was supported by the State Key Laboratory of Pulp and Paper Engineering (201502) and the Undergraduate Innovation and Entrepreneurship Training Program (201510057015).

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Article submitted: Oct. 29, 2015; Peer review completed: Jan. 14, 2016; Revised version received and accepted: March 16, 2016; Published: March 22, 2016.
DOI: 10.15376/biores.11.2.4146-4158