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Flow-through immobilization of *Candida rugosa* lipase on hierarchical micro-/macroporous carbon monolith

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ABSTRACT

Hierarchical micro-/macroporous carbon monoliths are prepared as enzyme carriers for flow-through process. The immobilization of *Candida rugosa* lipase on micro-/macroporous carbon monoliths is studied. Lipase is immobilized by physical adsorption which lipase solution is circulated through the micro-/macroporous carbon monolith. An accessibility of lipase to the surface inside the micro-/macroporous carbon monolith is enhanced by flow-through method which promotes enzyme–surface interaction and finally leads to rapid enzyme immobilization. After immobilization is conducted for 10 min, the maximum protein binding can be measured. In terms of substrate-immobilized lipase reactions, flowing of substrate through lipase immobilized micro-/macroporous carbon monolith promotes high efficiency in both reaction and product withdrawal. Moreover, at high flow rates of lipase solution in immobilization step, the lipase activity increases. Oxygenated surface of micro-/macroporous carbon monoliths support also demonstrates an interesting effect on lipase immobilization and biocatalyst activity. The initial reaction rate of lipase immobilized on oxygenated surface carbon monolith support has higher activity compared with normal surface.

1. Introduction

Immobilization of enzyme on several supports has been studied in order to improve the activity, stability, reusability and cost effectiveness of enzyme [1–3]. The selection of support materials for enzyme is a very important factor to prevent enzyme leaching in the process. Porous materials such as carbon, ceramic and polymer are widely used as supports in enzyme immobilization due to their high surface area and pore volume. Moreover, size and character of pore structure of these materials can be tailored made to be specific with enzymes [1,4–7].

For lipase immobilization, the hierarchical porous carbon is an attractive support. Carbon has hydrophobic surface which most lipase supports have been modified to be more hydrophobic in order to improve the activity of lipase [2,8]. Not only hydrophobic surface but also pore size and shape of the hierarchical porous carbon support have a big effect on lipase immobilization and activity [9]. In addition, functional groups on the surface of carbon support can be adjusted to suit lipase immobilization. Recently, Quirós et al. [10] reported that the oxygenated functional group on the surface of carbon supports prepared by plasma oxidation promotes enzyme adsorption and activities of immobilized lipase compared with normal surface.

Among various shapes of enzyme supports, bead supports have been commonly used in packed bed or stirred reactor [11]. However, monolithic porous support is an ideal support for flow-through process. From its low back pressure, good mass transfer and high throughput [12], monolithic porous support as been used as a stationary phase in continuous flow process. This material shows several advantages on many applications such as chromatographic support, separation membrane, effective reaction column and catalytic carrier [13–17]. Therefore, hierarchical porous carbon monolith has a great potential as enzyme supports in biocatalyst reaction under rapid flow conditions to increase the activity and reduce the reaction time.

In the present study, the immobilization of *Candida rugosa* lipase on a hierarchical porous carbon monolith with micropores on an interconnected macroporous skeleton synthesized from sol–gel polycondensation of resorcinol and formaldehyde is investigated. The effects of circulating flows and the flow rates of enzyme and substrate solutions as well as the effects of oxygenated surface of micro-/macroporous carbon monoliths on lipase immobilization

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are intensively discussed. An interesting point is in most lipase immobilization on porous materials, only mesoporous or meso-/macroporous materials are widely used as supports [10,18–20] while the micro-/macroporous carbon monolith is originally investigated in this study.

2. Experimental

2.1. Enzyme and chemical reagents

C. rugosa lipase (Type VII) and *p*-nitrophenyl palmitrate (*p*-NPP) are purchased from Sigma–Aldrich Co. Resorcinol ($C_6H_4(OH)_2$; 99%) is obtained from Fluka. Formaldehyde (HCHO; 36.5%) and nitric acid (HNO_3) are acquired from Ajax Finechem. 1-Butanol and *t*-butanol are analytical grades supplied by Carlo erba Co.

2.2. Synthesis of macroporous carbon monolith precursors

Resorcinol–formaldehyde (RF) solutions are prepared from resorcinol and formaldehyde in de-ionized water. Nitric acid solution 0.5 M is used as a catalyst in the synthesis of interconnected macroporous carbon monolith [21]. The molar ratios of resorcinol to formaldehyde (R/F), and resorcinol to water (R/W), and the mole to volume ratio of catalyst to water (C/W) are fixed at 0.50 mol/mol, 0.15 mol/mol, and 0.20 mol/ml, respectively. Firstly, resorcinol is dissolved into the de-ionized water and stirred until the dissolution is completed. Then, the resorcinol solution is added to formaldehyde solution and followed by adding 0.50 M nitric acid. All solutions are then kept in a thermostated bath. Before RF solutions lost their fluidity, they are transferred into the cylindrical glass tube where RF gels are formed. Next, RF gels are aged in hot air oven at 75 °C for 24 h. After aging, RF gels are removed from the tube and dried at 75 °C with hot air until constant weight is achieved. The hierarchical micro-/macroporous carbon precursor in monolithic form is finally obtained.

2.3. Preparation of hierarchical porous carbon monolith column

An activated RF carbon monolith (ACM) is prepared by carbonization under 50 cm^3-STP flow of N_2 gas in a quartz tube reactor. The sample is heated up to 850 °C at 10 °C/min heating rate and held at 850 °C for 30 min. While an oxygenated carbon monolith (OCM) is obtained by CO_2 activation which CO_2 gas is used instead of N_2 gas in carbonization process [22]. Before enzyme immobilization, the obtained cylindrical shape carbon monolith (8 mm in diameter and 6 cm in length) is clad by heat-shrinkable tubing. Then, the encapsulated carbon monolith is packed into a precut glass tube with epoxy glue.

2.4. Characterization of hierarchical porous carbon monolith

The cross-sectional macropore texture of carbon monoliths is determined by scanning electron microscope (SEM; JEOL, JSM-6700F). Macropore size distribution and macropore volume of the macroporous carbon monoliths are characterized by mercury porosimeter (Micromeritics, Pore-Sizer-9320). The micro-/mesoporous properties are obtained from the adsorption and desorption isotherms of N_2 at 77 K measured by adsorption apparatus (BEL, mini-BEL Sorp.). The surface functional groups are identified by Fourier transform infrared (FTIR) spectra using spectrometer recorder (PerkinElmer, 1615). A weight lost after carbonization of the obtained carbon monoliths, carbonization yield, is calculated.

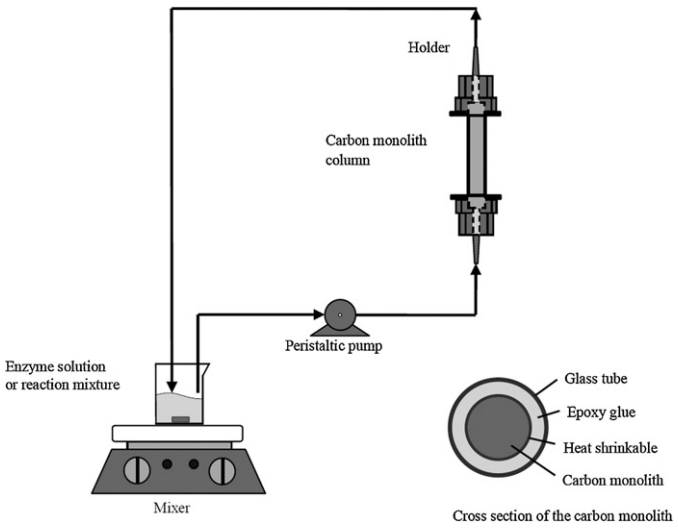

Fig. 1. Experimental set up for flow-through immobilization process by circulating lipase solution and reaction mixture in immobilization step and enzymatic reaction, respectively.

2.5. Lipase immobilization on the hierarchical porous carbon monolith column by flow-through method

The solution of 1 mg/ml lipases prepared in 20 mM phosphate buffer solution at pH 7 is circulated through the micro-/macroporous carbon monolith column by using peristaltic pump (Fig. 1). The lipase solution is circulated at 10, 22 and 55 ml/min flow rates for 120 min. After that, the lipase-immobilized carbon monolith column is three times washed with phosphate buffer to remove the unbound enzyme. The residual enzyme and washing solutions are recovered and analyzed for unbound protein. The lipase-immobilized carbon monolith column is dried in a desiccator and stored at 4 °C.

The protein binding efficiency is calculated as the amount of protein bound to the carbon monolith column by measuring the difference of protein between the initial and residual (including the washing solutions) protein concentrations in solution. The concentrations of protein before and after immobilization are determined by Bradford protein assay method [23].

2.6. Enzyme activity assays for lipase-immobilized carbon monolith

Transesterification between *p*-nitrophenyl palmitate (*p*-NPP) and 1-butanol is employed to determine an activity of lipase immobilized on micro-/macroporous carbon monolith [9]. The stock solution of *p*-NPP is prepared in *t*-butanol. The reaction mixtures (19 ml *p*-NPP solution and 1 ml 1-butanol) are circulated through the lipase-immobilized carbon monolith column at 10 and 55 ml/min flow rates for 180 min at room temperature. The sampling of 5 μl reaction mixture is collected about 20 times within 180 min. Each sampling is then mixed with 300 μl triethylamine–ethanol solution (3 μl triethylamine in 1 ml ethanol). The products (*p*-nitrophynol) formed by the enzymatic transesterification are spectrophotometrically determined at 410 nm.

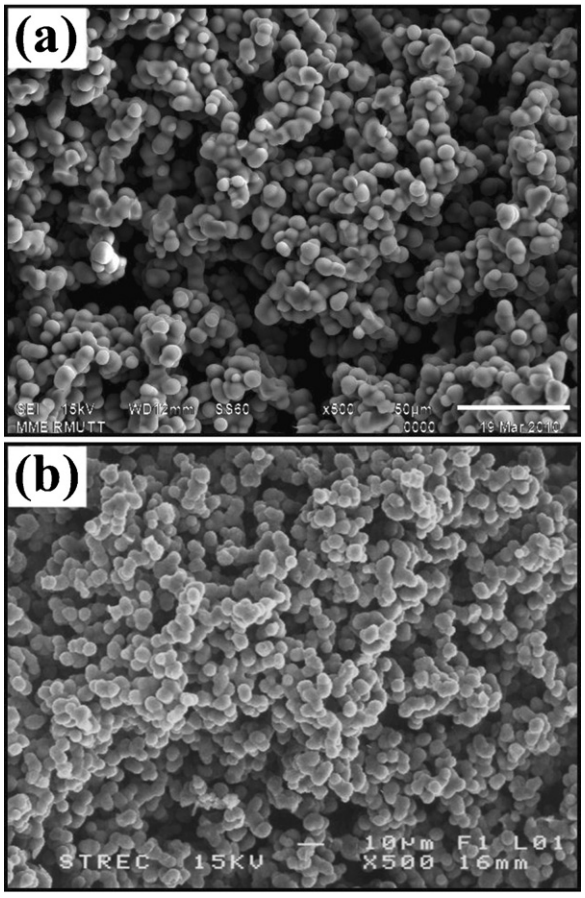

Fig. 2. SEM images show cross-section of the samples ACM (a) and OCM (b).

3. Results and discussion

3.1. Characterization of porous properties of hierarchical porous carbon monolith

Macroporous textures of hierarchical carbon monoliths are observed by SEM images as shown in Fig. 2. These SEM images confirm the appearance of interconnected macroporous structure of the activated carbon monolith (ACM) and the oxygenated carbon monolith (OCM). Macropore size distributions of carbon monoliths are shown in Fig. 3. It can be indicated that the macropore diameter of samples ACM is larger than OCM. Moreover, the macropore volume of OCM is slightly smaller than ACM. More shrinkage in OCM compared to ACM leads to smaller macropore diameter and volume in OCM corresponded with carbonization yield. From Fig. 4, N_2 adsorption–desorption isotherms of OCM and ACM are classified as type I microporous material by IUPAC. Micropores on macroporous skeleton of these carbon monoliths are clearly confirmed by porous properties from Figs. 3 and 4. According to Table 1, BET specific surface area (S_{BET}) and micropore volume (V_{mic}) of OCM are larger than S_{BET} and V_{mic} of ACM.

These results indicate that the macropore skeletons of ACM and OCM are similar as shown by SEM images. However, the macropore diameter of OCM is smaller compared with ACM whereas the surface area of OCM is larger. These results indicate that the CO_2 activation shows higher shrinkage of the carbon structure than carbonization with N_2 while higher surface area can be explained by an increase in micropore volume.

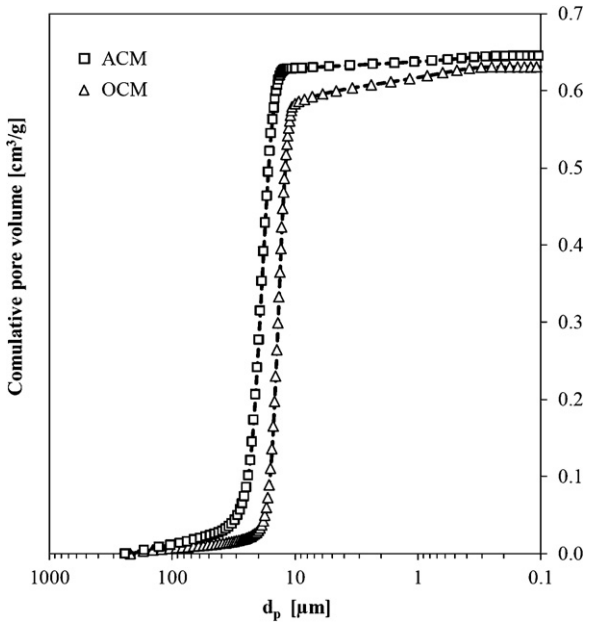

Fig. 3. Macropore size distributions of the samples ACM (□) and OCM (△).

3.2. Chemical surface property of carbon monoliths

Infrared spectra of carbon monoliths prepared by carbonization with N_2 (ACM) and by physical activation with CO_2 (OCM) are shown in Fig. 5a and b, respectively [24].

Infrared spectra of ACM are analyzed (Fig. 5a). It is clearly seen that only two peaks can be observed at all spectrum regions. The peaks at 1624 and 1335 cm^{-1} can be attributed to C=C stretching vibration and C—H bending vibration modes in basal plane, respectively. Hence, no functional groups on the surface of carbon monolith prepared by carbonization with N_2 (ACM) can be detected [25–27].

According to Fig. 5b, four significant peaks which can be observed at the peaks around 3439, 1630, 1051 and 586 cm^{-1} in the sample OCM. The peaks at 1630 and 586 cm^{-1} can be attributed to C=C stretching vibration modes and out-of-plane bending vibration of C—H, respectively, in the basal plane of carbon. In addition, the peak around 1630 of OCM is too sharp, it possible presence of C=O vibration. The C—OH stretching vibration peak is shown in

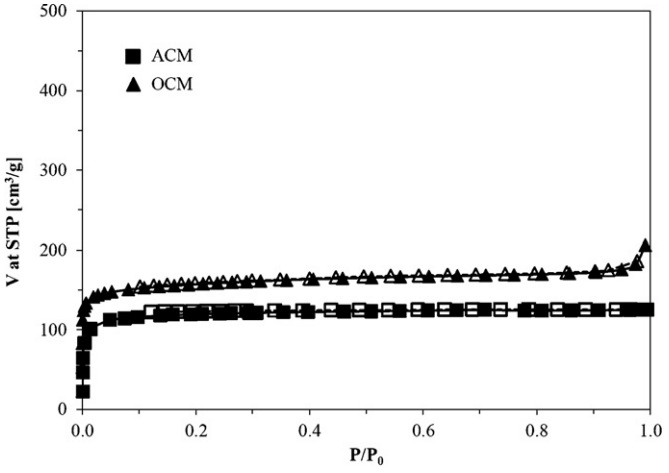

Fig. 4. N_2 adsorption–desorption isotherms at 77 K of the macroporous carbon monoliths prepared by carbonization with N_2, ACM and physical activation with CO_2, OCM.

Table 1
Porous properties of the micro-/macroporous carbon monoliths.

Samples	Carbonization yield[a]	Macropore properties		Micropore properties		S_{BET} [m^2/g]
		V_{macro} [cm^3/g]	Mean pore diameter [μm]	Type of isotherm	V_{micro} [cm^3/g]	
ACM	57	0.65	18.6	Type I	0.19	289
OCM	64	0.63	13.5	Type I	0.25	385

[a] Carbonization yield shows mass loss from sample in carbonization process which correspond with the development of microporosity of the carbon [22].

broad band at 1051 cm^{-1}. Moreover this peak is also attributed to the C—O—C stretching vibrations. Therefore, the presence of peak at 3439 cm^{-1} can be assigned to the subsistence of hydroxyl or phenol group on the surface of carbon monolith [25–33]. From these results, there is a high possibility that the oxygenated functional group on the surface of carbon monolith can be generated by CO_2 activation method.

3.3. Flow-through lipase immobilization

The immobilization of lipase is operated by circulating of enzyme solution through the oxygenated micro-/macroporous carbon monolith (OCM) packed in the glass column. The strong bonding between carbon particles on the skeleton of macroporous carbon monolith leads to no breakage of small carbon particles after the circulating flows of lipase and substrate solutions.

The activity of lipase solution is investigated within time interval to calculate the optimum immobilization time which expressed as the percentage of residual activity (Fig. 6). A decrease in lipase activity indicates that the attachment of soluble active enzyme on the surface of carbon monolith support decreases rapidly with an increment of immobilization time. The maximum active enzyme loading is achieved in approximately 10 min of immobilization process at the lowest of residual activity. High mass transfer and low diffusion resistance of macroporous carbon monolith strongly help lipase to rapidly diffuse into the inside surface of hierarchical porous carbon monolith. Lipase immobilization on micro-/macroporous carbon monolith by flow-through method results in the shortest immobilization time compared with other diffusion methods.

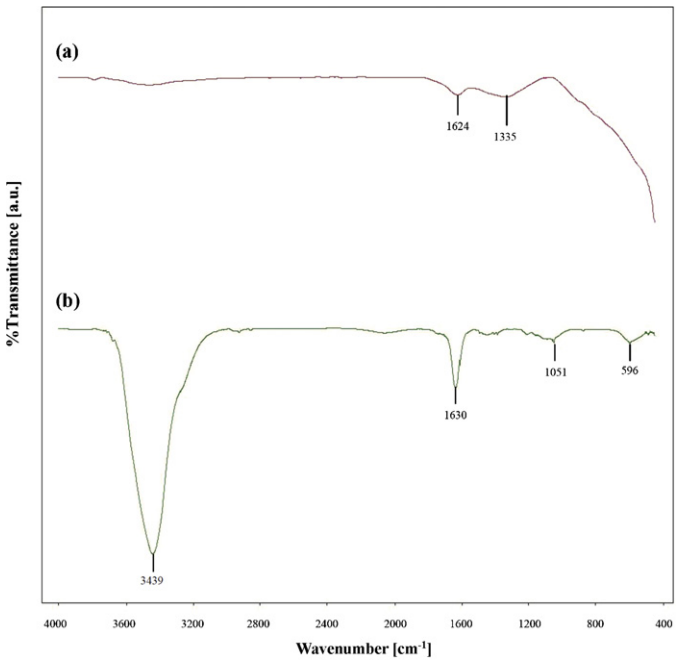

Fig. 5. FTIR spectra of the carbon monoliths obtained from carbonization with N_2, ACM (a) and physical activation with CO_2, OCM (b).

3.4. Effect of lipase solution flow rate and substrate flow rate on immobilized lipase activity

The effects of enzyme solution flow rate and substrate flow rate are studied by measuring immobilized lipase activity in terms of initial reaction velocity and protein binding efficiency (Table 2). The initial reaction velocity which is the rate of product generation related to the immobilized lipase activity. Although, the immobilized lipase catalyzes transesterification reaction and quantitatively provides same final cumulative products for all different flow rates, an increase in the flow rates of lipase solution in the immobilization process drastically increases the initial reaction velocity.

For the ACM column, the protein binding efficiencies of ACM-10, ACM-22 and ACM-55 columns are 56%, 58% and 54%, respectively. It is indicated that flow rate of lipase solution has no effect on the binding of enzyme on ACM support. However, the initial reaction velocity of ACM columns is slightly different at various immobilized flow rates. The maximum initial reaction velocity is obtained from ACM-55 column immobilized with 55 ml/min flow rate of lipase solution.

In case of OCM column, OCM-10 immobilized lipase has 71% protein binding efficiency which is higher than the columns immobilized with 22 and 55 ml/min flow rates (51% and 55% protein binding efficiencies, respectively), but the initial reaction velocity is lower. These results suggest that lipase immobilized at 10 ml/min flow rate has more time for structural rearrangement which is mainly protein–protein interaction so more protein bindings on the support lead to steric impediments; therefore, more enzymes attached on the surface of carbon do not correspond with their activity [19]. For OCM-22 and OCM-55 columns immobilized with 22 and 55 ml/min flow rates respectively, the initial reaction velocity on OCM-55 column is 3.3×10^{-4} μmol/min which is 2 times faster than OCM-22 column (1.7×10^{-4} μmol/min) while the protein binding efficiency is the same. It indicates that the high flow rate of enzyme solution plays a remarkable role in minimizing the

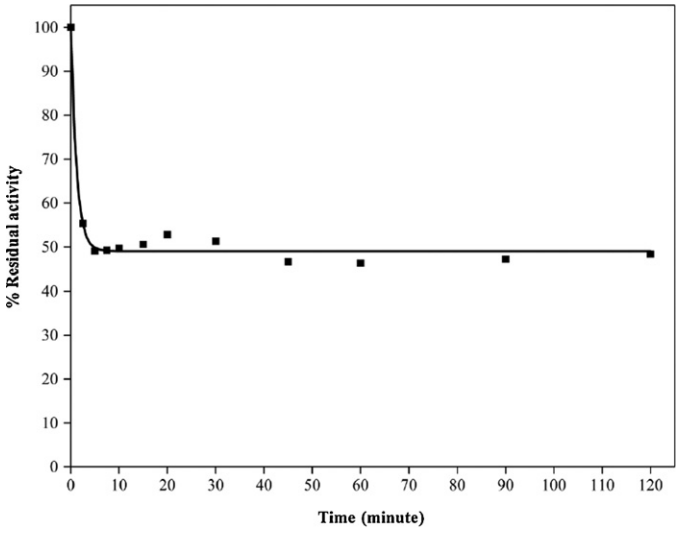

Fig. 6. %Residual activities in enzyme solutions at various immobilization times.

Table 2
Protein binding efficiency and initial reaction velocity of lipase-immobilized on the ACM and OCM columns at various flow conditions.

Columns	Flow conditions in the experiment		Protein binding efficiency (%)	Initial reaction velocity ($\times 10^4$ μmol/min)
	Enzyme solution (ml/min)	Substrate solution (ml/min)		
ACM-55	55	55	54	1.0
ACM-22	22	55	58	0.8
ACM-10	10	55	56	0.7
OCM-55	55	55	55	3.3
OCM-22	22	55	51	1.7
OCM-10a	10	55	71	1.5
OCM-10b	10	10	69	0.6

steric resistance of immobilized lipase in terms of improvement in enzyme distribution on the surface as a result the substrate easily reaches the active site of enzyme [10,34]. Therefore, the initial reaction velocity is increased when the immobilization is carried out at high flow rate.

As results, the protein binding efficiency of ACM and OCM columns are similar at 22 and 55 ml/min immobilized flow rates; although, surface area of OCM is larger. In this case, the attachment of enzyme on carbon support does not depend on the surface properties of materials at high immobilized flow rate. However, OCM column at 10 ml/min flow rate shows more protein loading compared with ACM column. In addition, the chemical and textural on surface of carbon support has an interesting effect on the initial reaction velocity. The initial reaction velocity of OCM columns is clearly higher than ACM columns at all immobilized flow rates. These results reveal a high possibility that lipase can be induced to active form when it is attached on oxygenated surface of OCM column.

The effect of substrate flow rate is studied by flowing of the substrate through lipase-immobilized carbon monolith column to catalyze the reaction. The production rate is determined by calculating the initial reaction velocity. The initial reaction velocity of OCM-10a column measured at 55 ml/min flow rate of substrate is 1.5×10^{-4} μmol/min which is 2.7 times higher than the initial velocity of OCM-10b column (10 ml/min flow rate of substrate), 0.6×10^{-4} μmol/min. As a result, high yield occurs at higher flow rate of substrate; although, the amount of protein binding on the supports is similar. It clearly suggests that the circulating flow of substrate through the lipase-immobilized micro-/macroporous carbon monolith outstandingly leads to good substrate–immobilized enzyme interaction when the substrate flow rate is increased, the products can also be easily removed from the macroporous skeleton of carbon monolith support. In summary, the flow rate of substrate in the reaction also plays an important role on the enzymatic activity because an accessibility of substrate to enzyme on the surface of micro-/macroporous carbon monolith support is easy and the production rate is increased at high flow rate of substrate.

3.5. Role of oxygenated surface of micro-/macroporous carbon monolith on kinetic parameters of immobilized lipase

Influence of physical surface treatment of micro-/macroporous carbon monolith on enzymatic mechanism is discussed. Immobilized lipase on ACM and OCM columns which have no surface functional group and surface functional groups, respectively are compared by immobilization at 55 ml/min flow rate. Kinetic parameters of transesterification activity of immobilized lipase on micro-/macroporous carbon monoliths are investigated by varying the initial substrate concentrations of *p*-NPP (10–50 mM). In Fig. 7, the plots of initial reaction velocity against the substrate concentration reveal the catalytic mechanisms of lipase immobilization on the oxygenated surface and untreated surface of

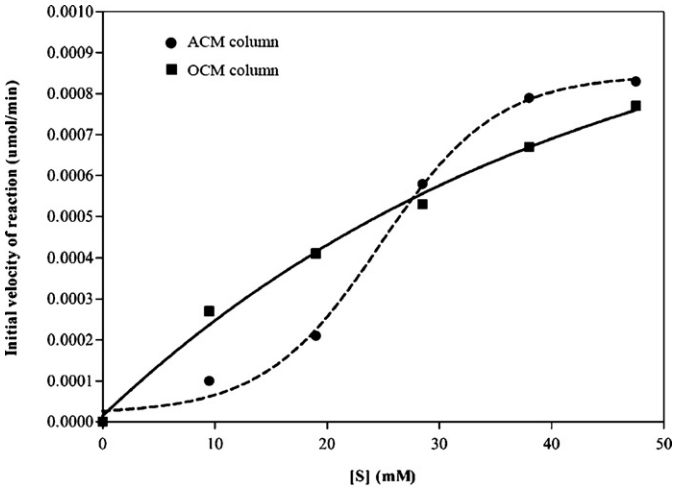

Fig. 7. The relationship between initial velocity and substrate concentration in transesterification reaction catalyzed by lipase-immobilized on the ACM (●) and OCM (■) columns.

micro-/macroporous carbon monoliths. The kinetic constant; affinity binding of enzyme toward substrate, K and the maximum velocity, V_{max} can be calculated by using enzymatic equations [35].

For catalytic mechanism, the lipase immobilization on the OCM and ACM columns show a single-substrate reaction and an allosteric reaction, respectively [35]. The estimated values of V_{max} and $K_{0.5}$ of immobilized lipase on ACM column are approximately 2 times lower than values of V_{max} and K_m obtained from OCM column (Table 3). Although, the protein binding efficiency of OCM and ACM columns are similar at high immobilized flow rate, the carbon monolith with oxygenated surface exhibits clearly higher efficiency compared with the untreated surface carbon monolith. Thus, the oxygenated surface of micro-/macroporous carbon monolith exhibits a better transesterification rate. An improvement in the transesterification rate can probably be explained by an induction of slightly polar substrate (*p*-NPP) by the oxygenated surface of micro-/macroporous carbon monolith.

Table 3
Kinetic parameter of lipase-immobilized on the ACM and OCM columns.

Columns	Protein binding efficiency (%)	K (mM)	V_{max} ($\times 10^4$ μmol/min)
ACM	50	26[a]	8.3
OCM	54	51[b]	15.6

[a] K is referred to $K_{0.5}$ since the kinetic results show the sigmoidal curve and a Hill equation is used to calculate kinetic parameters [35].
[b] K is referred to K_m since the kinetic results followed the Michealis–Menten equation [35].

4. Conclusions

Hierarchical micro-/macroporous carbon monolith is successfully used as a support for immobilized *C. rugosa* lipase by flow-through method. Flowing of lipase solution through the micro-/macroporous carbon monolith support is completed within 10 min of the immobilization process resulting from high mass transfer and low diffusion resistance of macropores, thus enzyme rapidly access into the surface inside the hierarchical porous carbon monolith support. Moreover, the flow rate of enzyme solution and oxygenated surface are a dominant factor. The activity of immobilized lipase on ACM and OCM columns is improved with an increasing flow rate of enzyme solution. In addition, the flow rate of enzyme solution in ACM column does not have significant effect on protein binding, but the protein binding on OCM column is developed at low flow rate. However, the presence of oxygen on the surface promotes higher efficiency than its activated carbon monolith counterpart although the protein binding is slightly different. Furthermore, the flow rate of substrate in the reaction plays an interesting role on enzymatic activity since it can lead to better accessibility of substrate to enzyme on the surface of micro-/macroporous carbon monolith support. In addition, the products can be easily removed via macropores of the carbon monolith support.

Micro-/macroporous carbon monolith is proved to be a potential support for lipase immobilization because of its hydrophobic surface, high surface area and surface modification besides its size and pore structure can be tailored made. Low back pressure, good mass transfer and high throughput are advantages of the micro-/macroporous carbon monolith which can be applied as a support for flow-through/continuous enzyme reaction or a component in a micro reactor.

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