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Design of large-pore mesoporous materials for immobilization of penicillin G acylase biocatalyst

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Abstract

In this study, functionalization of large-pore nanoporous silica materials was carried out by condensation of tetraethylorthosilicate (TEOS) and 3-aminopropyltriethoxysilane (APTES), 3-mercaptopropyltrimethoxysilane (MPTMS), phenyltrimethoxysilane (PTMS), vinyltriethoxysilane (VTES), and 4-(triethoxysilyl)butyronitrile (TSBN), respectively, in the presence of non-ionic surfactant under acidic conditions. The TSBN functionality was subsequently converted to carboxyl group while APTES was further functionalized with glutardialdehyde, a cross linker. The various functionalized materials were used as supports for immobilization of enzyme penicillin G acylase (PGA). Experimental data showed that the functionalized materials except for the material functionalized with MPTMS possess a faster loading kinetics and a higher loading amount of enzyme PGA than the pure-silica counterpart. The enzymatic catalytic activities of the immobilized biocatalysts varied from 52.2 to 167.5 U/g of solid. The glutardialdehyde-activated material displayed the highest initial immobilized enzyme activity and the most stable activity among all the support materials. PGA immobilized on VTES-functionalized nanoporous silica showed the highest initial enzymatic activity (67.7 U/mg of PGA, much higher than that of free PGA (300 U/mg of PGA). Experimental data along with theoretical analysis results indicate that glutardialdehyde is a good cross linker, offering covalent binding of PGA with the support materials while VTES-functionalized nanoporous silica is a very good potential support for physical entrapment of PGA enzyme.

Keywords: Functionalization of mesoporous silicas; Penicillin G acylase; Immobilization; Enzymatic activity

1. Introduction

Enzyme biocatalysts are finding increasing applications because of the growing demand for biotransformations with a high chemo- and stereospecificity in the fine chemicals industry such as pharmaceuticals, agrochemicals, and health care products. However, the application of an enzyme for a given reaction is often hampered by its reusability. This is because free enzyme, as a biocatalyst, is lacking of long-term stability under process conditions and difficult to recover and recycle from the reaction mixture, making the reuse of the enzyme impossible. Hence the idea of immobilizing the enzyme on a rigid solid support enabling easy separation and the possibility of operation in a packed-bed or fluidized-bed reactor has been of great industrial interest for many years [1]. In the past few decades, many immobilization methods and carrier materials have been investigated [1–3].

Organic polymeric carriers are the most widely studied materials because of the presence of rich functional groups, which provide essential interactions with the enzymes. For example, Eupergit C [4,5], oxirane acrylic beads, has been commercially used as an enzyme carrier. However, the organic supports suffer a number of problems such as poor stability towards microbial attacks and organic solvents, and disposal issues.

In contrast, inorganic materials such as silica gels, alumina, and layered double hydroxides are known to be thermally and mechanically stable, non-toxic, and highly resistant against microbial attacks and organic solvents [1]. Among them, porous inorganic carriers with a high specific surface area and a large-pore volume are a promising family of materials for enzyme immobilization. In addition to the common advantages of inorganic supports, the pores provide a higher density of enzyme loading and facilitate transport of substrate and product.

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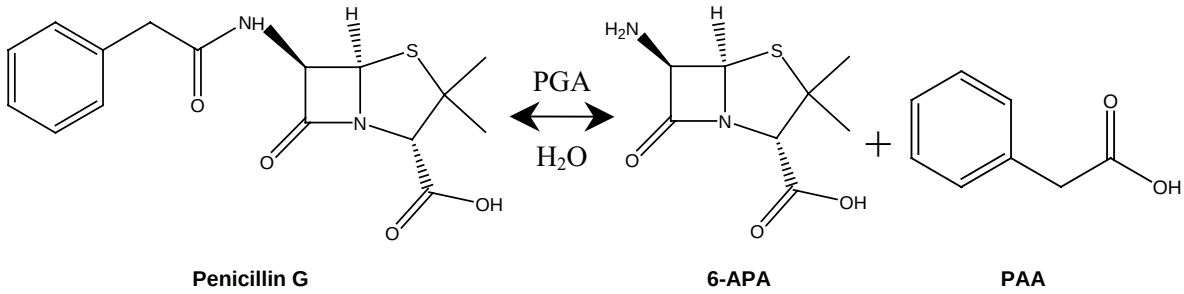

Fig. 1. Hydrolysis of penicillin G catalyzed by penicillin G acylase.

Since the availability of highly ordered nanoporous materials [6], immobilization of a number of enzymes on them has been demonstrated [7–20]. In most of the previous studies, reduced enzymatic activity upon immobilization was observed because of many reasons such as pore diffusion resistance. Interestingly, a recent study [18] showed that immobilized organophosphorous hydrolase on carboxyl-functionalized nanoporous materials displayed an enzymatic activity of two times higher than the free enzyme. The authors interpreted that the carboxyl functionality created a benign microenvironment for the particular enzyme. Yiu et al. [11–13] observed that the presence of organic functional groups on the surface of nanoporous SBA-15 minimized leaching of enzyme trypsin from the supports. The authors also observed that immobilized trypsin on thiol- and carboxyl-functionalized SBA-15 materials showed a good specific activity.

Penicillin G acylase (PGA) (E.C. 3.5.1.11.4) is an enzyme catalyzing the hydrolysis of penicillin G to produce 6-aminopenicillanic acid (6-APA) (see Fig. 1), which is a precursor for manufacturing some semi-synthetic antibiotics [4].

In this study, PGA was immobilized on nanoporous SBA-15 materials functionalized with thiol (–SH), phenyl ($–C_6H_5$), vinyl (C=C), amine ($–NH_2$) (which subsequently reacted with glutardialdehyde), and nitrile (C=N) groups (which was subsequently converted to COOH groups). The influence of the various supports on the loading of PGA and catalytic activity was studied. The reusability of the immobilized PGA biocatalysts was examined. Experimental and theoretical data showed that vinyl is an excellent functionality, which offers a synergic interaction with PGA while glutardialdehyde is a good cross linker, which provides covalent binding of PGA with the support materials.

2. Experimental

2.1. Chemicals

The chemicals used in this study were non-ionic triblock copolymer poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (P123, Aldrich), TEOS (98%, Acros Organics), APTES (99%, Aldrich), MPTMS (95%, Avocado), PTMS (97%, Aldrich), VTES (97%, Aldrich), TSBN (98%, Aldrich), glutardialdehyde (50% in water, Acros Organics), hydrochloric acid (37 wt.%, Merck), sulphuric acid (95–97%, Merck), NaOH solution (1N, Merck), Penicillin G Acylase (42 mg protein/mL, Sigma), potassium phosphate buffer (monobasic anhydrous, Sigma), Bradford reagent (Sigma), and Penicillin G (benzylpenicillin, Sigma). All the chemicals were used as-received without further purification.

2.2. Preparation of support materials

A pure-silica SBA-15 was prepared according to Zhao et al. [21]. The synthesis of amine-, thiol-, phenyl-, vinyl-, and nitrile-functionalized SBA-15 samples was essentially similar to that of the pure-silica SBA-15 sample except for adding a certain amount of an organosiloxane. The P123 template was removed from the functionalized samples by using ethanol extraction at 70 °C for 6 h. This was repeated three times to ensure complete removal of surfactant P123, which was confirmed by thermogravimetric analysis. The functionalized samples are denoted as APTES-silica, MPTMS-silica, PTMS-silica, VTES-silica, and TSBN-silica, respectively.

Functionality of APTES was further treated with glutardialdehyde in order to enable cross linkage of PGA with the support as schematically shown in Fig. 2. About 1 g of APTES-functionalized silica sample was combined with 100 mL of 2.5 % (v/v) glutardialdehyde solution at pH 7.0 and room temperature under stirring for 1 h. The solid was then filtered off and washed with deionized water. The glutardialdehyde-activated support is denoted as Silica-Glutar. Functionality of TSBN was subsequently converted to carboxyl (COOH) functional groups according to the method described by Ho et al. [22]. The sample is designed as silica-COOH.

2.3. Characterization of support materials

The physical properties of the support materials were characterized by using small-angle X-ray scattering (SAXS), N_2 sorption, transmission electron microscopy (TEM), and

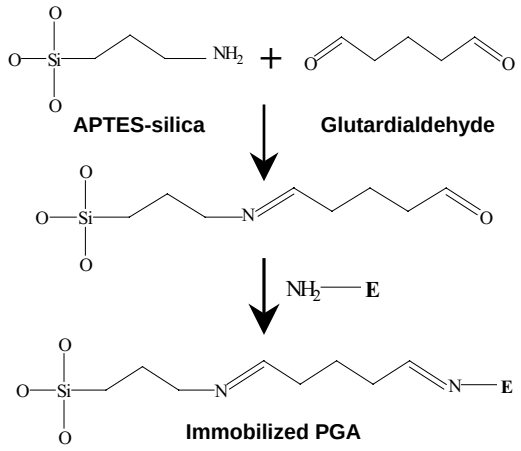

Fig. 2. Schematic representation of covalent binding of enzyme PGA with glutardialdehyde cross-linker, which is chemically bonded to amine functionality on silica support.

scanning electron microscope (SEM) techniques. The details of characterization techniques can be found elsewhere [23,24].

2.4. Kinetics of immobilization of PGA on supports

The loading kinetics of PGA on the various supports was measured as follows. PGA was first diluted by combining 0.032 mL of enzyme solution (42 mg/mL) with 5.96 mL of 50 mM potassium phosphate buffer with a pH 6.0. About 0.1 g of support solid was immersed into 6.0 mL of the diluted enzyme solution of pH 6.0 at room temperature under shaking in a water bath at 200 rpm (GFL1092). The 100 μL of filtered supernatant was taken every 1 h and then subjected to Bradford assay [25] for a continuous duration of 24 h. A UV-1601 spectrophotometer (Shimadzu) operated at a wavelength of 595 nm was used to determine the concentration of PGA in the supernatant.

2.5. Immobilization of PGA on supports

PGA immobilization was carried out as follows. About 0.5 g of a support material was combined with 30 mL of the diluted enzyme solution (42 mg/mL). The mixture was shaken at room temperature for 12 h at 200 rpm. The solid was filtered off, washed with deionized water, and dried at room temperature for 20 min. The loading amount of PGA was calculated according to the concentration differences before and after 12 h shaking measured by using Bradford assay [25].

2.6. Measurement of enzymatic activity

Enzymatic catalytic activities of the immobilized biocatalysts were determined by titrating phenylacetic acid (PAA), a by-product of reaction of hydrolysis of Pen-G (see Fig. 1), with 0.5N NaOH solution to maintain a constant pH of 7.8. An automatic titrator (Metrohm 718 Stat Titrino) was used to carry out the continuous titration at 37 °C, which was maintained by using a circulated water bath. A concentration of 2% (w/v) of the substrate was used to evaluate the initial catalytic activities of the biocatalysts. Kinetic study was carried out on selected samples in the substrate concentration range of 0.02–12.5% (w/v). Eq. (1), the Michaelis–Menten (M–M) kinetic model was used as Eq. (2) to linearize the reciprocal of initial activity, $1/-r_s$, versus the reciprocal of the substrate concentration, $1/C_s$, to obtain constants K_m and V_{max}:

$$-r_s = \frac{V_{max} C_s}{K_m + C_s} \quad (1)$$

$$\frac{1}{-r_s} = \frac{K_m}{V_{max} C_s} + \frac{1}{V_{max}} \quad (2)$$

The substrate and products of the hydrolysis reaction were analyzed by using a high-performance liquid chromatography (HPLC) (Hewlett-Packard HP 1100) equipped with a wavelength-variable UV detector. An ODS Hypersil column with dimensions of 250 mm × 4.6 mm was used at 30 °C. A mixture containing 50 mM of 84% (v/v) potassium phosphate buffer with a pH of 6.0 and 16% (v/v) of HPLC grade acetonitrile (Fisher Scientific) was used as the mobile phase with a flow rate of 1 mL/min, operated at a wavelength of 225 nm. Standard 6-APA (96%, Acros Organics) and Pen-G potassium salt (Sigma–Aldrich) were used for calibration purposes. The HPLC elution curves of the products catalyzed by immobilized PGA on pure-silica SBA-15, a mixture containing substrate (pure Pen-G) and 6-APA, and pure 6-APA are shown in Fig. 3. The elution times for 6-APA and PAA were observed to be 2.7 and 3.8–4.1 min, respectively. A very small amount of substrate Pen-G, which eluted in the time period of 10–12 min, was also observed from the reaction system.

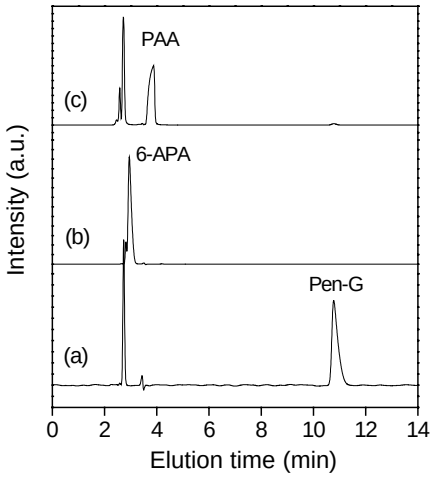

Fig. 3. HPLC elution curves: (a) standard penicillin G, (b) standard 6-APA, and (c) the liquid phase of the hydrolysis of penicillin G catalyzed by PGA immobilized on pure-silica SBA-15.

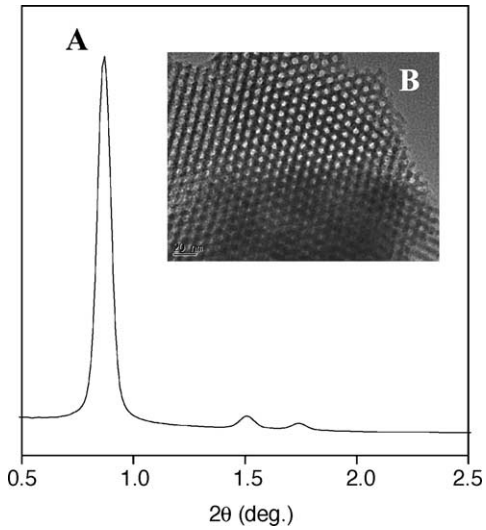

Fig. 4. SAXS pattern (A) and TEM images (B) of sample VTES-silica.

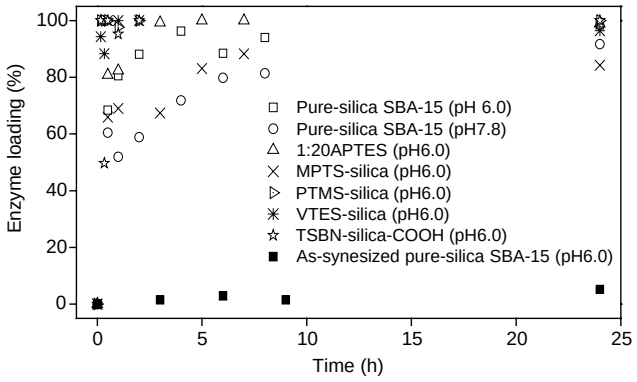

Fig. 5. Enzyme adsorption kinetic curves on pure-silica SBA-15, functionalized SBA-15, and as-synthesized SBA-15.

3. Results and discussion

3.1. Characterization of the support materials

The representative SAXS pattern and TEM image of sample TEVS-silica are shown in Fig. 4. The physical properties derived from SAXS and nitrogen adsorption measurements of the support materials are presented in Table 1. Structural analyses based on SAXS patterns showed that the support materials are nanoporous structures with a 2D hexagonal *p6mm* symmetry, i.e. SBA-15 materials [21]. The data in Table 1 show that the support materials have a pore size in the range of 60–90 Å. With considering the pore sizes and the PGA molecular dimensions of 70 Å × 50 Å × 55 Å [26], it is believed that the enzyme have been mainly immobilized on the pore surfaces instead of on the external surfaces.

3.2. Adsorption kinetics and loading amount of PGA on the supports

Adsorption kinetics under two pH values (pH 6.0 and 7.8) were measured on the pure-silica SBA-15 sample. From the adsorption kinetic curves shown in Fig. 5 it is seen that the uptake of PGA on the pure-silica SBA-15 at pH 6.0 was higher than that at pH 7.8. As a result, adsorption kinetic measurements for the functionalized samples were carried out at pH 6.0. It is also seen from Fig. 5 that all functionalized samples possess a higher enzyme loading and a faster adsorption rate than pure-silica SBA-15. Most of the functionalized SBA-15 showed an enzyme uptake of 80–100% in 0.5 h of incubation, whereas the pure-silica SBA-15 sample adsorbed only about 70% of PGA. Adsorption of PGA on an as-synthesized pure-silica SBA-15 sample (before template removal) was less than 5%, indicating that the majority of the enzyme was loaded on the interface surfaces of the support materials.

The time required for immobilization of PGA on SBA-15 materials was found to be shorter than that on MCM-41 materials [15]. After less than about 0.5 h of incubation, adsorption equilibrium was researched on the functionalized SBA-15 supports except for sample MPTS-silica, which required a much longer equilibrium time, about 5 h, similar to that equilibrium time of PGA on the pure-silica SBA-15 sample. The slow loading kinetics of PGA on MPTS-silica sample is believed to be due to the smallest pore size of MPTS-silica among the functionalized samples. The relatively small pore size of sample MPTS-silica imposed a high resistance to the diffusion of PGA, thus slowing down the adsorption rate. Nevertheless, to ensure adsorption equilibrium, 12 h was used for the subsequent experiments of immobilization of PGA.

Table 1
Physical properties of support materials

Sample	d_{100} spacing (Å)	S_{BET} (m^2/g)	D_{BJH} (Å)	V_{Pore} (cm^3/g)
Pure-silica SBA-15	97.1	854	90.0	1.20
APTES-silica	99.3	599	90.3	1.08
Silica-Glutar	–	466	89.8	0.802
MPTMS-silica	97.1	791	66.2	1.08
PTMS-silica	95.0	584	76.1	1.00
VTES-silica	102	555	89.8	1.00
TSBN-silica	103	715	89.7	1.12
Silica-COOH	102	478	108.0	1.02

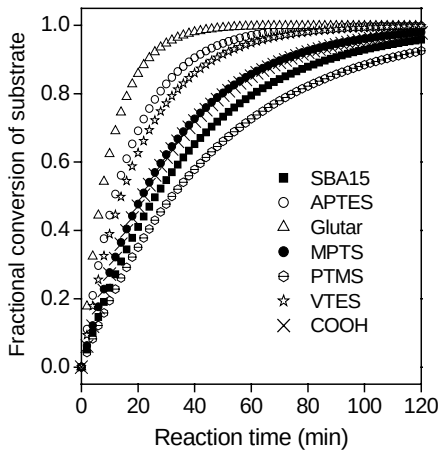

Fig. 6. Enzymatic activity of immobilized PGA on various functionalized supports.

3.3. Enzyme activity assay

In the design of enzyme support materials, enzyme activity after immobilization is one of the most important parameters for assessing the efficiency of the supported enzyme. A high loading of enzyme does not necessarily mean a high enzymatic activity because of the following reasons: (1) some of the active sites of the enzyme could be blocked upon immobilization [27,28], (2) immobilization via covalent binding will result in structure rigidity of the enzyme, causing difficulties for the enzyme to induce fit to the substrate [29], (3) conformational changes may occur or the active sites may be involved in binding to the support material [27], and (4) diffusion resistance to the substrate may result in an observed slow reaction rate [27].

Fig. 6 shows the normalized activity curves (on a 0.5 g IME basis) of the immobilized enzyme biocatalysts. It can be seen that the biocatalysts immobilized on all of the functionalized SBA-15 supports except for on sample PTMS-silica displayed a higher enzymatic activity than that on the pure-silica SBA-15 sample. The glutardialdehyde-activated support enabled the maximum conversion of Pen-G in about 40 min. However, PGA immobilized on the pure-silica SBA-15 support required about 120 min to reach the maximum conversion of Pen-G.

To compare the performance of the immobilized biocatalysts, the formation rate of product 6-APA, in the first 10 min was used to indicate the initial activities expressed as E_{IME} (U/g) and E_{Enzyme} (U/mg), respectively. The former term stands for the initial activity of a whole immobilized enzyme system while the later one represents the initial activity of the enzyme upon immobilization on the solid carrier. E_{Enzyme} enables one to better compare the enzymatic activity of the enzyme after immobilization with its soluble form (free enzyme). The initial activity of free PGA was experimentally measured to be 30 U/mg enzyme, agreeing very well with that reported by the supplier. The initial activities of the biocatalysts are reported in Table 2.

Very interestingly, it can be seen from Table 2 that in despite of a moderate value of E_{IMEs}, the E_{Enzyme} of sample VTES-silica, which was calculated to be 67.1 U/mg enzyme, is two times higher than that of the free enzyme, showing that vinyl is a very effective functionality for immobilization of PGA biocatalyst. The observed high E_{Enzyme} on support VTES-silica also indicates that the enzyme immobilized on this material maintained a stable conformation structure, a flexible mobility of the active sites, negligible blocking effect of the active sites, and a high accessibility of the active sites to the substrate. Such an improved enzymatic performance is most likely due to the enhanced surface hydrophobicity because of the presence of vinyl groups, which induced a higher affinity of the substrate to the immobilized enzyme. Thus, the attractive forces of the support towards the substrate were enhanced, leading to an increased reaction rate. However, it should also be noted that a highly hydrophobic surface may also have an adverse effect on the enzymatic performance of an immobilized enzyme because the active sites of the enzyme are believed to line up with hydrophobic residues [28,29], leading to blockage of the active sites by the support. In addition, the hydrophobic forces may also cause conformation changes of the enzyme. Nevertheless, when only a low amount of vinyl groups was on the surface of VTES-silica material, such adverse effects can be ignored.

On the other hand, sample Silica-Glutar exhibited the highest value of E_{IME}. However, immobilization of PGA on this support caused about 30% decrease in E_{Enzyme} in comparison with the free enzyme and about 12% decrease in E_{Enzyme} in comparison with PGA immobilized on the pure-silica SBA-15 sample. This observation indicates that a high loading of enzyme does not necessarily afford a high E_{Enzyme} as some of the supported enzyme may be inactive. Previous studies [2,30,31] have demonstrated that an immo-

Table 2
Summary of activities of enzymes immobilized on different support materials[a]

Support	Enzyme loading (%)	Enzyme loading ($\times 10^{-13}$ molecule/m^2)	E_{IME} (U/g)	E_{Enzyme} (U/mg)	Activity decrease (%)
SBA-15	89.8	17.43	62.3	23.7	76.8
APTES-silica	95.9	30.61	119.4	17.4	83.2
Silica-Glutar	94.1	28.01	167.5	20.8	28.1
MPTMS-silica	85.8	15.49	74.3	23.7	43.7
PTMS-silica	97.2	10.81	52.2	22.0	79.0
VTES-silica	~100	9.34	104.3	67.1	51.6
Silica-COOH	84.2	20.59	73.1	21.0	61.2

[a] E_{Enzyme} = 30 U/mg enzyme for free enzyme PGA, which was confirmed experimentally.

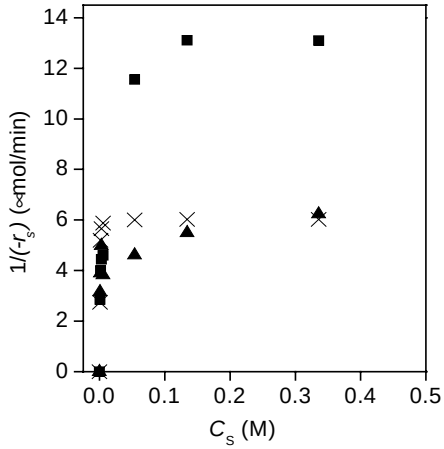

Fig. 7. Hydrolysis rate of penicillin G acylase catalyzed by immobilized PGA biocatalysts: (■) PGA immobilized on Silica-Glutar; (×) PGA immobilized on pure-silica SBA-15; (▲) PGA immobilized on VTES-silica.

bilized enzyme tends to have a lower E_{Enzyme} than its soluble form, especially if the covalent coupling method is employed. Glutardialdehyde, a cross linker, enabled covalent linkage of the support through amino groups of APTES and the $–NH_2$ groups of PGA (see Fig. 1) or perhaps the single catalytic active site of N-terminal serine on chain B of PGA [24]. Thus, PGA immobilized on sample Silica-Glutar is more rigid than on the rest of the samples and some of the active sites could have been lost. This is believed to be the main reason of causing the reduction of E_{Enzyme} according to the enzyme–substrate induced fit theory [27].

Because of immobilization of PGA on a porous solid support, pore diffusion resistance may have a significant effect on the intrinsic rate described by Michaelis–Menten kinetic model, thus causing a small particle effectiveness factor (η) [13,15,16,32]. In the present study, particles of micrometer size were used. Thus, we were confident that mass transfer effects can be ignored [33]. Fig. 7 shows the observed reaction rates of hydrolysis of Pen-G as a function of substrate concentration catalyzed by three representative immobilized biocatalysts, namely PGA immobilized on pure-silica SBA-15, on Silica-Glutar, and on VTES-silica, respectively. By plotting $1/-r$ versus $1/C_s$, values of K_m (Michaelis constant) and V_{max} (maximum rate of reaction) were determined and are reported in Table 3. According to the M–M equation, V_{max} is dependent on the amount of immobilized enzyme while K_m is a reciprocal indicator of substrate–enzyme affinity [34]. To evaluate the efficiency of the immobilized enzyme systems, an efficiency index (V_{max}/K_m) was used and is reported in Table 3 as well. It can be seen that the kinetic parameters agree with the initial activities and the enzyme loadings discussed earlier. Sample Silica-Glutar with a high loading of PGA showed the highest value of V_{max} and K_m among the three immobilized enzyme systems. Sample VTES-silica with the highest E_{Enzyme} exhibited the lowest K_m. The efficiency index suggests that sample VTES-silica is the most efficient carrier for immobilization of PGA among the functionalized supports in this study.

Table 3
K_m and V_{max} values for the immobilized PGA biocatalysts

Support	K_m ($\times 10^3$ M)	V_{max} (μM/min)	Efficiency index, V_{max}/K_m ($\times 10^3$)
SBA-15	0.675	6.53	9.67
Silica-Glutar	1.16	8.35	7.20
VTES-silica	0.339	5.07	15.0

K_m value is inversely proportional to the affinity of substrate to the enzyme.

3.4. Recycle and reuse of the immobilized PGA biocatalysts

The reusability of an immobilized enzyme makes the effort of immobilization of enzyme worth it. Therefore leaching or deactivation of the immobilized enzyme is a major problem encountered in biocatalysis. Enzyme leaching is common for immobilized enzymes. In the present study, PGA was immobilized on most of the supports via adsorption except for on support Silica-Glutar. Nevertheless, the E_{IME} of the biocatalysts immobilized via physical forces was still acceptable with about a 28% decrease in the second run. The biocatalyst immobilized on pure-silica SBA-15 suffered severe desorption of enzyme, which caused a decrease of about 76.8% of E_{IME} in the second run. The functional groups of –SH, $–CH_2{=}CH_2–$, and COOH improved the stability of the immobilized PGA compared to pure-silica SBA-15, whereas $–NH_2$ and $–C_6H_6$ functionalities did not show any improvement in terms of the stability of the immobilized PGA. However, it should be noted that the decrease in E_{IME} of the immobilized biocatalysts could also be due to the inactivation of enzyme upon immobilization such as product and/or substrate inhibition [34].

4. Conclusions

It can be concluded that the presence of certain functionalities on nanoporous SBA-15 supports can improve their properties for immobilization of enzyme PGA. The pore size of the support materials is important in determining the loading rate and capacity of PGA on them. However, as observed in this study, a high loading amount of PGA does not necessarily mean a high enzymatic activity. Many factors have influences on the performance of immobilized PGA biocatalyst.

Glutardialdehyde, a cross linker, can dramatically improve the stability of immobilized PGA because of the formation of covalent binding, thus minimizing enzyme leaching. However, it must be pointed out that enzyme immobilization via covalent binding may reduce the enzyme mobility to induce fit to a substrate, causing difficulties in regeneration of the immobilized enzyme biocatalyst.

Vinyl-functionalized SBA-15 silica offers a high initial enzymatic activity and a low K_m value among the functionalized supports in this work. The hydrophobic interactions between the enzyme and vinyl functional groups are the primary forces for binding PGA. In addition, the hydrophobic surface properties of the support material may play an important part in attracting the substrate to approach the enzyme, thus enhancing the enzymatic activity.

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