Biocatalytic Conversion Efficiency of Steapsin Lipase Immobilized on Hierarchically Porous Biomorphic Aerogel Supports

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ABSTRACT: Hierarchically porous aluminosiloxane aerogels (ALS-PG) with a rare structural architecture were developed through a biotemplating method using pollen grains of Hibiscus rosa-sinensis. The unique structure of the Hibiscus rosa-sinensis pollen makes it an attractive biotemplate, by replicating all levels of macro- and mesoscale morphological features. The micromorphological analysis exposed funnel-shaped macrochannels between the mesoporous aerogel framework that are difficult to design artificially. The N2 sorption analyses confirmed hierarchical trimodal pore size distribution with an average mesopores diameter (ca. 3.9, 8.7, 26.6 nm), high BET/Langmuir surface area (497/664 m2 g−1) and large pore volume (1.6788 cm3 g−1) than the corresponding nontemplated aerogels and aerogels counterpart. Beneficial properties of this sophisticated hierarchical porous structure was examined and confirmed by the immobilization of steapsin lipase. Hierarchically porous ALS-PG showed enhanced loading and immobilization efficiency (32.3 mg g−1 and 74.21%) when compared to non templated ALS-WO-PG (11.2 mg g−1 and 41.40%). It was further improved with the methyl (MTMS@ALS-PG) (69.8 mg g−1 and 96.87%) and amino propyl (APTMS@ALS-PG) (65.1 mg g−1 and 94.96%) surface modifications. Additionally, it showed enhanced catalytic performance for hydrolytic, esterification, and transesterification reactions. It is anticipated that this hierarchically porous aerogel supports can suitably hold the biocatalyst and can solve critical problems associated with its native state for technological applications.

KEYWORDS: Hierarchical porosity, Alumino-siloxane supports, Immobilization, Steapsin lipase, Biocatalysis

INTRODUCTION

Ever growing demand for next generation “clean and green fuels” strongly merits the production of biofuel from biomass via biocatalytic processes.1−3 However, biocatalyst (or enzyme) mediated biofuel production is facing a technological hitch with respect to the high cost of enzymes, their low storage, operational and thermal stabilities.2−3 When these enzymes are used as such in the free-state, their separation is obligatory to avoid contamination of the product. Moreover, the enzymes are lost after the first use which again leads to additional cost. Immobilization of biocatalysts in robust thermochemically stable support is indeed a promising alternative to overcome this technological challenge.1−3−5 It calls for new and efficient immobilization methods that further demand the development of exceptional support/carrier material.

In the past decade, many attempts were reported for the immobilization of enzymes in porous and nonporous supports; both organic and inorganic. In majority of the studies, powdered nanomaterials were considered and explored due to excessive surface area and distinctive surface morphology.6−8 However, efficient separation of nanomaterial is again a major challenge for the enzyme recovery and its reusability for subsequent cycles. Hence, the use of monolithic carriers was another advancement made in this line. It offers assorted advantages such as ease of separation, rapid termination of reactions and adaptability to various engineering design for batch and continuous operations. To date, numerous efforts have been devoted to the development of monolithic organic polysaccharides and microporous polymers such as cellulose, calcium alginites, κ-carrageenan, chitosan (biopolymers),9−12 polyacrylic, polystyrene, polypropylene (synthetic polymers)1,13 microbeads for enzyme immobilization. However, the size, shape and strength of these organopolymeric carriers get altered upon repeated use. They also show weak resistance to pH, temperature, chemical corrosion and are largely prone to microbial contamination.

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Notably, attention turns toward the development of monolithic ceramic porous supports. They have merits such as thermal and mechanical stabilities, nontoxicity and high resistance to organic solvents/microbial attack. Indeed, porous inorganic supports such as zeolites,\(^{14}\) aluminum phosphates,\(^{15}\) alumina/silica gel,\(^{13,15}\) and unimodal mesoporous silica (M41S/SBA-n)\(^{6,13,15}\) have been investigated for enzyme encapsulation and immobilization. The presence of well-defined porosity in such inorganic porous material favors high enzyme loading. Among these supports, aluminum phosphates and zeolitic materials cause serious diffusion limitation due to their small pore diameter in micropore domain.\(^{16}\) In the case of ordered mesoporous materials, the problems associated with pore diffusion is minimal, but the lack of macroporosity hinders the transport phenomena.\(^{17−19}\) Nowadays, there have been attempts to make hierarchically porous materials with different length scales of porosity integrated in a single porous solid. They have the potential to provide high diffusion and superior mass/heat transfer characteristics.\(^{16−20}\) Moreover, the presence of multidimensional and multidirectional pores can enhance the inclusion of large biomolecules (enzyme loading), immobilization rates and favor better transport of substrate and product.

Hence in the present report, we demonstrate the design of hierarchically porous alumino-siloxane (ALS) inorganic aerogels having ingenious structural architecture through a biotemplating method. Until now, considerable work has been reported on the synthesis of hierarchically porous materials via templating approach using colloidal crystal, polymer latexes particles, silica spheres, emulsion droplets, surfactants micelles, gas bubbles, poly(methyl methacrylate) and polystyrene microspheres.\(^{18,20,21}\) However, all these templates are either uneconomical for practical applications due to its complex preparation process or often require tedious procedures for its removal from the matrix. Thus, the biotemplating approach is an environmentally benign and inexpensive route to engineer hierarchically porous functional material. Nature has excellent microarchitectures with precise widths and lengths, sophisticated exterior and interior surfaces and uniform geometries, which have inspired researchers to explore them as "replica/templates" to generate multiscale porous materials. Many researchers have skillfully exploited materials such as cottonfibers,\(^{22}\) legume fruits,\(^{23}\) butterfly wings,\(^{24}\) pollen grains\(^{25}\) and microorganisms (bacteria and viruses)\(^{26}\) to produce multiscale hierarchical porosities. In this work, we explored ubiquitous and inexpensive natural pollen grains of Hibiscus rosa-sinensis for the first time as the biotemplate to create hierarchical porosity in ALS gels. Hibiscus rosa-sinensis is one of the readily available and widely grown ornamental plants throughout the tropics and subtropics. Normally, its pollen grains have specific surface morphology, uniform particle size and are easy to harvest and store.

We have recently reported the synthesis of thixotropically reversible ALS gels to form aerogels and xerogels microbeads.\(^{27}\) Here, we attempted to build on our previous work, by engineering multilevel porosity through inherited pore morphological features of natural pollen grains, which are implausible to produce artificially. Most importantly, the hierarchically porous ceramic ALS aerogel supports were explored for steapsin lipase immobilization for biocatalytic application.

Lipase enzymes (triacylglycerol ester hydrolases, EC 3.1.1.3) have emerged as one of the leading biocatalysts for a range of biochemical transformations; esterification, hydrolysis, aminolysis, and transesterification reactions.\(^{3,4,5,7}\) A variety of lipases of microbial origin (Candida, Rhizopus, Pseudomonas, Bacillus, Aspergillus, Penicillium etc.) have been immobilized in various supports and investigated previously.\(^{5,7}\) Steapsin lipase, a known digestive enzyme found in pancreatic juice, was chosen in this study. From the literature, we acquainted that immobilization of steapsin lipase on porous ceramic gel supports is not much investigated. In this work, hierarchically porous ceramic aerogel microbead was developed as a host material for steapsin lipase. Subsequently, the effect of aerogel structure on loading and enzymatic activity of steapsin lipase was investigated in detail.

For any support material, the surface properties are decisive for the binding ability and performance of the respective biocatalyst. It often requires surface modification.\(^{28}\) In our work, we also adopted surface modification strategy and studied the role of functional groups and their binding sites interactions with steapsin lipase to have enhanced loading capacity and immobilization efficiency. In order to illustrate the broad applicability of the immobilized steapsin lipase, its thermal, mechanical and storage stabilities and organic solvent tolerance ability were compared with free enzyme. Finally, in this research, biocatalytic efficiency of immobilized steapsin lipase was tested for the hydrolysis, esterification, and transesterification reactions.

This work is an overall assessment of the unique structure of the Hibiscus rosa-sinensis pollen grain for the creation of hierarchically porous architectures and also the demonstration of the efficiency of such porous architecture for immobilizing steapsin lipase for biocatalytic application.

**EXPERIMENTAL SECTION**

**Materials.** Aluminum isopropoxide (AIP, purity >98%), 3-aminopropyltrimethoxysilane (APTMS, purity >99%), methyltrimethoxysilane (MTMS, purity >98%), 4-nitrophenylopalmitate (pNP) and olive oil were purchased from Aldrich. 25% ammonium solution and parafl in liquid light was obtained from Merck Specialties Pvt. Ltd. Glutaraldehyde (GA) (25% in water) was obtained from Spectrochem Pvt. Ltd. Natural pollen grains of Hibiscus flowering plant (Hibiscus rosa-sinensis) (Figure 1a–c) of the family Malvaceae, were collected from the local area. Steapsin lipase (activity ≥40–70 U/mg) was procured from Sisco Research Laboratories. The Bradford reagent was purchased from Bio-Rad Laboratories Pvt. Ltd. Nitric acid, isopropanol, hexane, methanol, ethanol, butanol, toluene, acetonitrile, isopropanol, hexane, methanol, ethanol, butanol, toluene, acetonitrile,
overnight incubation at room temperature. The supports were then referred to as MTMS@ALS-PG. A total of 1 g of MTMS or APTMS modiﬁcation regarding the MTMS or APTMS concentrations (Scheme 1). The supports were washed and dried in a vacuum oven at 100 °C for 10 h to remove water molecules adsorbed on the surface. These wet gel microbeads were then converted to aerogel microbeads as per the procedure described in our previous report with some modiﬁcations27 (details are given in S2). These pretreated PG were used for the experiment. Uniform spherical particles of pretreated PG have an average diameter of 100–105 μm, with spiky exine/spines of height and width 12 and 5 μm, respectively. In a typical procedure, 0.25 g of pretreated pollen grains was dispersed in magnetic stirred ALS gel. After 30 min of stirring, pollen grains were homogeneously distributed in the ALS gel. So formed ALS-PG gel was then injected into a chemical bath of ammonium/parafﬁn oil mixture. Self-assembly of the gel in the oil layer forms well-deﬁned microbeads due to the surface tension. These wet gel microbeads were then converted to aerogel microbeads as per the procedure described in our previous work27 (details are given in S3). For comparative studies, we have also prepared corresponding xerogel samples. For this, wet gel microbeads were washed and dried in an oven at 60 °C for 12 h. The dried aerogel/xerogel microbeads were ﬁnally calcined @ 600 °C for 12 h. The resulted and the spectrophotometric absorbance of the supernatant was measured at 405 nm. One unit of steapsin lipase activity (U) was deﬁned as the amount of the enzyme, which catalyzed the production of 1 μmol of p-nitrophenol from pNPP per min under the test conditions. The protein binding yield of immobilized steapsin lipase was determined by Bradford methodology at 595 nm.30 The immobilization efﬁciency of supports was evaluated in terms of protein loading capacity (Q), immobilization yield (IY) and enzyme activity (EA) as follows:

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Q (\text{mg/g support}) = \frac{[(C_0 - C)]V}{M} \\
IY (\%) = \frac{(A - B)}{A} \times 100 \\
EA (U/g support) = \frac{(A - B)}{M}
\]

Where, C0 and C are the initial and ﬁnal enzyme concentrations in the solution (mg/mL), V is the volume of the solution (mL), M is the mass of the support (g), A is the activity of enzyme added in the initial solution, and B is the total activity of residual enzyme in immobilization and washing solution after immobilization process. By taking the maximum activity value of the immobilized and free lipase under optimal conditions to be 100%, the activities obtained from other conditions were expressed as relative activities. The relative
Biocatalytic Applications. Biocatalytic efficiency of immobilized steapsin lipase was validated using hydrolysis, esterification and transesterification reactions (detailed procedures of the reactions are given in S5 and Scheme S1).

Characterization. Physical, chemical and surface properties of the porous gel supports fabricated in the present study were systematically characterized using several instrumental techniques. (Details of the technique used for characterization are given in S6.)

RESULTS AND DISCUSSION

Characterization of ALS-PG Aerogel Microbeads. To determine the thermal behavior and calcination temperature, TGA/DTA was performed with pure pollen grains (PG), aerogel supports embedded with (ALS-PG) and without (ALS-WO-PG) pollen template (Figure 2a). It was observed that

pure PG undergoes stepwise thermolytic degradation, followed by an absolute decomposition with a strong exothermic DTA peak at the temperature range 580–600 °C. The difference in weight loss observed between ALS-WO-PG and ALS-PG revealed the loaded amount of PG, in ALS gel matrix is ca. 14 wt %. Further, TGA/DTA of as-synthesized ALS-PG showed three stages of weight loss at 50–120, 120–270 and 280–600 °C (Figure S1). The first weight-loss region below 120 °C is associated with desorption of residual and adsorbed water (ca. 7.3%), corresponding to an obvious endothermic peak in the DTA curve. Second weight loss (ca. 5%) between 120 and 280 °C may be attributed to the burning and removal of sacrificial pollen template as well as fragmentation of the organic framework forming a sharp endothermic peak at 276 °C. Subsequently, the third more pronounced weight loss (ca. 19%) observed between 280 and 600 °C with a spiky exothermic peak at 420 °C, which can be attributed to Si−OH and Al−OH condensation and combustion of residual organic matter. This was followed by complete removal of the remaining organic residues, and the weight loss reaches at equilibrium after 600 °C. The photographs (Figure 2b) of ALS-PG supports calcined under different temperatures also substantiate the complete decomposition of all organic residues from the gel matrix at 600 °C. Thus, based on this result, we choose 600 °C as the calcination temperature to remove the organic residue and sacrificial template to built exhilarating pore architecture in ALS aerogel matrix.

XRD results of uncalcined and calcined (at 600 °C) ALS-PG aerogel samples are shown in Figure 2d. The uncalcined ALS-PG showed a convoluted XRD pattern of broad bands at 13.2°, 28.1°, 38.2°, 49.4° and 65.0°, which denotes the nanometric scale of the boehmite (JCPDS card no. 21-1307)31 amalgamated with a broad peak at 21° due to the siloxane counterpart.32 The XRD pattern of calcined ALS-PG aerogels showed three very weak and broad peaks at 20 values 22°, 45° and 66° corresponding to a lower degree of crystallinity. From XRD results, we confirmed that the molecular-scale mixing of two metal-oxide precursors inhibited the preferential crystalline growth of any transition alumina during calcination and resulted in homogeneous amorphous Al−O−Si framework. Further, the EDS spectra (Figure 2c) shows three main peaks correspond to Al, O and Si signifying the existence of Al−O−Si framework in ALS-PG aerogel. The relative atomic percentage of Al, O and Si was found to be 15.19, 14.08 and 70.72, respectively (Figure 2c, inset). The elemental mapping using the EDS attachment on SEM was also performed. The elemental mapping corresponding to the areas is depicted in three different colors for Al (red), O (blue) and Si (green) signals (Figure 2e), which revealed the spatial uniformity of the elemental distribution in ALS-PG porous gel matrix.

The ALS-PG aerogel microbeads remained intact with no shrinkage or breakage of the gel framework after calcination at 600 °C (Figure 3a). The evidence for the structure engineered hierarchical macro-scale porosity in ALS-PG aerogel supports is acquired from micro morphological analysis. Figure 3a–g depicts the intriguing multilevel porosity generated by replication of hibiscus pollen microspheres. From Figure 3c,d, it can be clearly seen that the replica of the exact morphology and characteristic surface features of the sacrificial PG templates was faithfully inherited on the surface of gel framework. Furthermore, the hard spiky exine of the PG (shown in the inset of Figure 3d) created large funnel-shaped pores with external diameter of 5–6 μm having 10–12 μm cone depth (Figure 3d,e). The magnified images in Figure 3f,g shows the presence of well-defined macropores on the wall of the large conical pores. Further, the higher magnification SEM image (Figure 3b) shows that the gel framework is composed of closely packed nanoporous structure constructed with many interconnected nanowalls between the macropores. Finally, from TEM image (Figure 3i) it was perceived that the primary building units of the gel framework are composed of nanofibrillar structures which are entwined to form nanoporous framework.

The hierarchical mesoscale porosity was further confirmed by nitrogen sorption experiment. We observed a remarkable difference in porosity and physical features of the developed ALS-PG aerogel supports when compared to its xerogel counterpart (obtained by evaporative drying). Representative nitrogen adsorption/desorption isotherms and the corresponding pore-size distribution (inset) are shown in Figure 4. The ALS-PG aerogel supports show adsorption/desorption isotherm between type IV and type II (Figure 4a), characteristic of
the mesoporous material, according to the IUPAC classification.33,34 The isotherm exhibits a combination of H1 and H3 type hysteresis loop, with a very steep rise in adsorbed nitrogen at high relative pressure \((p/p_0 > 0.85)\), indicating the presence of secondary porosity of very large mesopores having a narrow slit or cylindrical shape pore geometries.33,34 On the other hand, their xerogel counterpart showed type IV isotherm with H2 type hysteresis loop, indicating the presence of small mesopores with ink bottle shaped pore structures (Figure 4b).27,34 In addition, a notable difference is observed in BJH pore-size distribution curve (PSD) (1−50 nm) (inset Figure 4a). The ALS-PG aerogel supports showed hierarchical trimodal PSD with small mesopores (ca. 3.9 and ca. 8.7 nm) and large mesopores (ca. 26.6 nm). ALS-WO-PG aerogels showed only a bimodal PSD (ca. 5.5 and ca. 12.1 nm), which indicates that the inclusion of biotemplate enlarges the size of mesopores. However, in their xerogel counterpart only monomodal PSD with small mesopores ca. 6.8 nm for ALS-PG and ca. 4.2 nm for ALS-WO-PG was observed (Figure 4b inset).

The detailed physical and textural features of the porous gel supports are depicted in Table S1. The drying shrinkage and bulk density of the aerogels samples were significantly less when compared to its xerogel counterpart. This signifies the improved porosity in the aerogel microbeads. Similarly, the ALS-PG aerogel exhibited a higher specific surface area of 497 m² g⁻¹, and pore volume 1.6788 cm³ g⁻¹ than that of ALS-WO-PG which has a specific surface area 329 m² g⁻¹ and pore volume 1.4156 cm³ g⁻¹. Moreover, aerogel supports showed considerably higher specific surface area and pore volume when compared to its xerogel counterpart ALS-WO-PG (192 m² g⁻¹ and 0.7522 cm³ g⁻¹) and ALS-PG (236 m² g⁻¹ and 0.9589 cm³ g⁻¹), respectively.
From the above discussion, it can be concluded that the ALS-PG possesses exhilarating hierarchically engineered porous structure, with multilevel porosity in macro and mesoscale (Scheme 2a–c). The hierarchically porous structure can remarkably raise the surface active sites and provide easy and quick access of guest molecules to the interior of the material (Scheme 2D). Therefore, it is believed that this multilevel hierarchically porous microstructure can allow better penetration of bulky biomolecules or proteins. Moreover, it can reduce the diffusion restrictions and pore-plugging often encountered in monomodal porous structure studied earlier.17–19

Modification of the Porous ALS-PG Aerogel Supports.

Besides porosity, surface properties govern the overall performance of the material.6–8 Therefore, surface modifications were carried out to facilitate effective surface interaction and response required for a particular application. To verify the successful functionalization on ALS-PG aerogel supports, FTIR spectroscopic analysis was performed. The representative FTIR spectra of the unmodified and modified supports are shown in Figure 5. Prior to functionalization, the spectra displayed a broad prominent peak in the range 3700–3000 cm\(^{-1}\) (centered at 3448 cm\(^{-1}\)), clearly revealing the presence of numerous surface hydroxyl groups (Al–OH and Si–OH) on the surface of ALS-PG. In all the spectra, a broad absorption in the range 1200–900 cm\(^{-1}\), indicates the presence of asymmetric stretching of Al–O–Si at 921 cm\(^{-1}\) and Si–O–Si at 1080 cm\(^{-1}\) of the gel structure.25,32 The gel framework also possesses Si–O–Si symmetric stretching and bending vibrations at 799 and 471 cm\(^{-1}\), respectively.32 The MTMS modification was well indicated by the absorption at 1269 and 769 cm\(^{-1}\), which are assigned to the Si–CH\(_3\) symmetric bending and rocking vibrations, respectively.35 Similarly, the 2971 and 2835 cm\(^{-1}\) bands are observed due to asymmetric and symmetric stretching vibrations of –CH\(_2\), respectively. Further APTMS modification was indicated by peaks at 1639 and 1487 cm\(^{-1}\), corresponding to N–H bending and C–N stretching vibration of aminated APTMS@ALS-PG.15,35 The absorption of the –NH\(_2\) bending/scissoring vibration at 1564 cm\(^{-1}\), as well as the asymmetric and symmetric C–H bond (–CH\(_2\)–) vibrations were observed at 2925 and 2872 cm\(^{-1}\), respectively, also indicating the presence of aminopropyl moiety on APTMS@ALS-PG.15,27 The GA cross-linked APTMS@ALS-PG exhibits strong bands at 2948 and 2857 cm\(^{-1}\) ascribed to aldehyde C–H and alkyl C–H stretching vibrations, respectively.15 Similarly, the C═O and C═N stretching modes were detected at 1720 and 1642 cm\(^{-1}\) in GA@APTMS@ALS-PG.15 This confirmed the successful cross-linking of GA on APTMS@ALS-PG.

In the same way, the results of nitrogen physisorption (Figure S2a) also provide a hint on successful modification and demonstrate the pore blocking effects caused by modifiers. The slight reduction of the mass specific parameters (surface area and pore features) (Table S2) is due to the added mass and occupied space of the organic residues, which indicates the successful grafting of respective modifiers. The reduction follows the order ALS-PG > MTMS@ALS-PG > APTMS@ALS-PG > GA@APTMS@ALS-PG, with increasing chain length of the respective modifiers, also gives the proof for surface grafting. As expected, specific pore diameter also shows a trifiling decrease with the introduction of larger organic moieties, indicated by the decreasing height of the capillary condensation step (Figure S2b). Even though functionalization caused slight variations in pore parameters, multiporosity of various length scales and mesoscopic structure of the support remained intact.

To evaluate hydrophobicity/philicity of functionalized ALS-PG, powder contact angle was measured, and the results are summarized in Figure S2c. The largest contact angle of 109° among the samples tested is observed for MTMS@ALS-PG, which can also be validated from the photograph showing contact angle of a water droplet over the aerogel microbeads. The surface hydrophobicity decreases in the order of MTMS@ALS-PG > APTMS@ALS-PG > GA@APTMS@ALS-PG > ALS-PG, according to contact angle measurements. Similarly, zeta potential measurements were performed as a function of pH in order to determine the isoelectric point (IEP) and surface charge of the supports (Figure S2d). The IEP point of ALS-PG, MTMS@ALS-PG, APTMS@ALS-PG and GA@APTMS@ALS-PG are 5.9, 3.1, 9.9 and 4.4, respectively. Out of all the supports, APTMS@ALS-PG possesses positive charged surface over a wide range of pH (1–9.9) due to the presence of positively charged aminopropyl groups. A marked variation in IEP of all the support suggests that the electrokinesis surface properties are mainly governed by the functional groups attached on the surface. No considerable change was observed in IEP after exposure of the support in...
strong acidic and basic medium, which confirms the chemical stability of the functionalized support.

In summary, all these results confirm the successful anchoring of various functional groups on porous aerogel support. In the following section, the immobilization ability of steapsin lipase on the hierarchically porous aerogel support is discussed.

Optimization of the Steapsin Lipase Immobilization Conditions. The immobilization capacity of the porous gel supports was evaluated by investigating the amount of steapsin lipase loading. Significantly higher amount of protein loading and immobilization yield were observed in aerogel supports, than in their xerogel counterpart (Table S3 and Figure S3a). Most of the earlier reports showed a direct influence of pore diameter on enzyme immobilization process. In general, for an efficient loading of protein the pore void must be larger than the enzyme dimension (i.e., in the range 5–50 nm). Steapsin lipase belongs to the class of pancreatic globular protein, having a molecular mass of 35–50 kDa and an average molecular diameter of about 4–6 nm. The hydrodynamic diameter measured for steapsin lipase in phosphate buffer at pH 8 is ca. 5.4 nm. Probably for xerogels, the smaller mesopore diameter (4.2 nm for ALS-WO-PG and 6.8 nm for ALS-PG) with tight pore junctions inhibit the diffusion of guest molecules, leading to very negligible amount of protein loading (Table S3). However, in aerogel supports having larger mesopores the loading amount was enhanced to 11.2 mg g⁻¹ in AS-WO-PG, and it was further enhanced to 32.3 mg g⁻¹ in ALS-PG. Evidently, in ALS-PG aerogel supports, presence of multi-porosity with additional funnel-shaped macroradicals, provide a better accessibility to the mesoporous aerogel framework. This enhances the loading rate and facilitates the transport of relatively large steapsin lipase molecule into the mesoporous aerogel framework.

From most of the previous reports, we realized that the surface characteristic of the support has a strong influence on protein loading. To improve the surface characteristics of ALS-PG aerogel support, postsynthetic surface modification was carried out via silanization using organosilane (MTMS and APTMS). Initially, we investigated the optimum amount of modifiers required to obtain maximum protein loading, immobilization efficiency and enzyme activity for the functionalized supports. The results obtained were shown in Table S3. When 15% (w/v) MTMS and 10% (w/v) APTMS were used, the highest amount of enzymatic activity were observed for immobilized steapsin lipase. Additionally, GA has been used as a cross-linker on APTMS@ALS-PG (samples modified with 10% w/v APTMS) for binding enzymes in which the amino groups of steapsin lipase are expected to form a Schiff base with the aldehyde group of GA. The optimal GA concentration was found to be 0.5% (v/v) for the surface activation of GA@APTMS@ALS-PG supports. The results summarized in Table S3 indicate that the protein loading, immobilization efficiency and enzymatic activity for porous aerogel support follow the order MTMS@ALS-PG > APTMS@ALS-PG > GA@APTMS@ALS-PG > ALS-PG.

The improvement in immobilization capacity of functionalized supports was attributed to the physical and chemical changes occurred at the surface during functionalization. The immobilization process is mainly governed by different interactions between enzyme and support: covalent bonding, electrostatic forces, hydrophobic interactions, hydrogen bonding and van der Waals forces. These interactions depend upon the surface characteristic of the support. The highest enzyme activity was observed for MTMS@ALS-PG (971.72 U/g support). This is attributed to the strong interaction of the steapsin lipase with the surface hydrophobic group of MTMS@ALS-PG (Scheme 3b). Since, lipase enzymes are known to have a unique property called “interfacial activation”, in which active site of the lipase is covered by flexible region called “lid”. During the interaction with the hydrophobic surface, conformational changes occur with the opening of lid to make the active
The hydrophobic domains around the active site of steapsin lipase form a strong hydrophobic interaction between the closely packed arrays of methyl group on the surface of MTMS@ALS-PG. Such interaction stabilizes the conformation of steapsin lipase via "lid opening" and favors the active site's accessibility to substrates, and show excellent enzymatic activity. Because APTMS@ALS-PG also shows more or less similar enzyme activity (967.26 U/g support), it is obvious that surface hydrophobic interaction is not the only factor that can improve the enzymatic activity. Several other interactions on the surface can also contribute to increase the enzymatic activity. Although APTMS@ALS-PG is expected to possess hydrophobic character contributed by propyl group, the effect would be counterpoised by the hydrophilic amino group. In APTMS@ALS-PG, the charge determining species on the surface are largely protonated amine, which shifts the IEP to the basic region (IEP ca. 9.9) and proves it to be a basic support. Hence, the strong positive charge on the surface of APTMS@ALS-PG support (as observed in ζ-potential curve Figure S2d) can deliver a strong electrostatic interaction with the negatively charged steapsin lipase (IEP ca. 4.8 Figure S4) at pH of immobilization (Scheme 3c).

Further, GA was activated on the surface of APTMS@ALS-PG as a cross-linking agent for plausible covalent attachment with steapsin lipase (Scheme 1d). It was expected to react with the −NH₂ group on APTMS@ALS-PG forming imine bonds, leaving the terminal −CHO group for reacting with the −NH₂ residues of the enzyme (Scheme 3d). However, in this case, a marginal increase in loading amount (34.2 mg g⁻¹) was observed when compared to ALS-PG. It was not much appreciable when compared to that of MTMS@ALS-PG and APTMS@ALS-PG. This might be due to the decrease in pore diameter and pore volume of the support as a consequence of surface functionalization. Moreover, the covalent anchoring might have caused severe decrease in enzymatic activity (701.96 U/g support) probably due to orientation or restricted motion of enzyme and resulted in biased deactivation of enzyme.40

Whereas in pure ALS-PG aerogel support −OH group present on the surface is expected to interact with −NH₂ and −COOH group of the enzyme via hydrogen bonding (Scheme 3a). The hydrogen bonding is considered to have relatively weaker interaction than compared to hydrophobic and electrostatic interactions.40 Consequently, higher extent of immobilization was obtained when functionalized support was used, and follows the order: MTMS@ALS-PG > APTMS@ALS-PG > GA@APTMS@ALS-PG > ALS-PG.

To determine the efficient relationship between enzyme and support, various parameters that influence the activity of the immobilized enzyme were systematically studied. The dimension of the microbeads strongly affects the enzymatic activity and determines its suitability for engineering enzymatic reactor configuration.4,10 As shown in Figure S3b immobilized aerogel microbeads with average diameter of 0.5, 1, 2 mm showed only a slight variation in the enzymatic activity, while a drastic decrease was observed when it was 4 mm. Hence, with the increase in microbead size the activity of immobilized steapsin lipase decreases, which is similar to the observation reported in polymeric microbeads.10 This is mainly due to the substrate diffusion limitation, i.e., the large dimension microbeads have longer substrate diffusion distance which leads to decrease in activity of immobilized steapsin lipase. From the above findings, it can be concluded that the aerogel microbeads of 0.5−2 mm offered lesser diffusion resistance compared to the larger one.

The effect of pH on enzymatic activity is shown in Figure S3c. It was observed that the optimal pH of the free steapsin lipase showing maximum activity is ca. 7.0 and fluctuated within a very narrow pH range (Figure S5a), whereas the optimum pH for ALS-PG, MTMS@ALS-PG and GA@APTMS@ALS-PG is ca. 8. APTMS@ALS-PG showed excellent adaptability in a wider pH range, especially in the alkaline range 6−10. Probably, the shift in optimal pH for immobilized steapsin lipase is due to its stabilization in porous support, which improved its tolerance at high pH conditions.41 The reason for higher activity of APTMS@ALS-PG in a wide range of pH is not completely understood, perhaps the wide range of positive surface charge attracts more OH⁻ ions around the immobilized enzyme. This results in the partitioning of protons between the bulk phase and microenvironment of immobilized steapsin lipase, which eventually leads to higher immobilization capacity at a wider pH range.

Figure S3d shows the effect of reaction temperature on the catalytic activity of the free and immobilized steapsin lipase. For free steapsin lipase, the optimal reaction temperature was 35 °C and showed a higher extent of deactivation at a temperature beyond 45 °C (Figure S5b). Whereas the optimal enzymatic temperature for all the immobilized steapsin lipase was ca. 40 °C and exhibited excellent adaptability in a wider temperature range 35−50 °C. Higher enzymatic activity at a broader range of temperature could be attributed to the strong interaction of steapsin lipase with the functional groups on the support, thereby preventing thermal denaturation and enhancing the adaptability of immobilized steapsin lipase. However, immobilized steapsin lipase in all the modified supports showed the similar trend at the studied temperatures, indicating that
Operational Stability of Immobilized Steapsin Lipase. Thermostability. Figure 6a,b shows the thermal stability of free and immobilized steapsin lipase incubated in PBS at 45 and 50 °C, respectively. Free and immobilized steapsin lipase retained reasonably high activity at 45 °C compared to those at 50 °C. However, at both temperatures free steapsin lipase is inactivated at a faster rate than that of immobilized one. The deactivation follows the order; free steapsin lipase > ALS-PG > GA@APTMS@ALS-PG > MTMS@ALS-PG > APTMS@ALS-PG at both the tested temperatures. The free steapsin lipase lost about 50% of its initial activity at 45 °C and completely lost at 50 °C in 60 min of incubation time. Whereas the immobilized steapsin lipase retained more than 80% of the initial activity in 60 min and ca. 50% of its activity was retained after 10 h of incubation. We observed rather high activity retention in APTMS@ALS-PG than in MTMS@ALS-PG. Probably at elevated temperatures free steapsin lipase is inactivated at a faster rate than that of immobilized one. The deactivation follows the order; free steapsin lipase > ALS-PG > GA@APTMS@ALS-PG > MTMS@ALS-PG > APTMS@ALS-PG at both the tested temperatures. The free steapsin lipase lost about 50% of its initial activity at 45 °C and completely lost at 50 °C in 60 min of incubation time. Whereas the immobilized steapsin lipase retained more than 80% of the initial activity in 60 min and ca. 50% of its activity was retained after 10 h of incubation. We observed rather high activity retention in APTMS@ALS-PG than in MTMS@ALS-PG. Probably at elevated temperatures, the electrostatic interaction stabilizes the conformational mobility of immobilized steapsin lipase in a better way compared to hydrophobically bonded steapsin lipase. Overall, the result showed an enhanced thermostability of immobilized steapsin lipase in porous aerogel samples compared to its free state.

Organic Solvent Stability. Table S4 shows the results of stability of immobilized steapsin lipase in different organic solvents having varying polarity/dipole moment. It was observed that immobilized enzyme could retain above 50% of its activity in all the test solvents, except in butanol. Unfortunately, we could not exactly correlate the activity recovery of immobilized steapsin lipase with the polarity (log P)/dipole moment of tested organic solvent. The less-polar solvents (n-hexane and toluene) were found to be more promising solvents showing good tolerance ability when compared to polar one, which is similar to the observation in recent studies. Reports suggest that polar solvents can cause stripping of bound water from the surface of the enzyme and result in conformational distortion and inactivation of immobilized enzyme. While, nonpolar solvents often enhance the enzyme resistance against inactivation by holding the bound water layer on the surface. Similarly, here we also noticed that hydrophobic interface of MTMS@ALS-PG showed hyperactivation of steapsin lipase by opening the lid as observed previously and stabilizes the enzyme conformation under various solvents.

Mechanical and Storage Stability. The mechanical and storage stability of immobilized steapsin lipase in MTMS@ALS-PG and APTMS@ALS-PG was compared with free steapsin lipase. The mechanical stability was evaluated by vigorously shaking (500 rpm) the immobilized and free steapsin lipase for 10 days in PBS (Figure 7a). Free steapsin lipase lost its activity roughly in 7 days under vigorous shaking. Whereas immobilized steapsin lipase could retain 80% of its activity for the same period and ca. 50% of activity loss was only observed after 10 days of continuous shaking. Hence, the activity recovery for immobilized steapsin lipase is attributed to its firm binding with the support matrix. Similarly, storage stability was investigated at 30 °C for 30 days (Figure 7b). As speculated, immobilized supports showed better storage ability than the free one and retained 60–70% of its activity after 30 days. Therefore, the inherent mechanical property of porous supports play an important role in improving the mechanical and storage stabilities of immobilized steapsin lipase.

Figure 7. (a) Mechanical and (b) storage stabilities of free and immobilized steapsin lipase.

Figure 8. Time course of the (a) hydrolysis of olive oil in PBS (pH 8.0) and (b) esterification of oleic acid with methanol.
**Reusability.** Reusability is the key advantage of the immobilized enzymes compared to the free one. The variation in activity of the immobilized steapsin lipase after multiple cycles is illustrated in Figure S6. It was observed that the residual activity of the immobilized steapsin lipase remains high (above 90%) in the first 3 cycles for all the supports. Then, the catalytic performance of steapsin lipase in unmodified ALS-PG sharply decreased with the increase in cycle number. This might be due to weak physical bonding which leads to the leakage of the enzymes from the supports during reusing. While functionalized supports (MTMS@ALS-PG and APTMS@ALS-PG) retained ca. 50% of their activities even after 10 consecutive reuse. However, the residual activity of the immobilized steapsin lipase in GA@APTMS@ALS-PG fails to retain its activity after consecutive reuses. Unfortunately, no explanation could be predicted for such a drastic activity reduction in GA@APTMS@ALS-PG on reuse.

To justify our claims, we further compared the immobilization efficiency of the developed supports with some standard materials (Table S5). The results clearly indicate the beneficial effect of hierarchically porosity and surface functionalization in ALS-PG aerogel supports over other standard supports when used for enzyme immobilization process.

**Biocatalytic Performance of Immobilized Steapsin Lipase.** Biocatalytic performance of free and immobilized steapsin lipase was validated by hydrolysis, esterification and transesterification reactions (Scheme S1). As seen from Figure 8a, the yield of free fatty acids (FFA) released were 81%, 24%, 76% and 62% when free, immobilized steapsin lipase in ALS-PG, MTMS@ALS-PG and APTMS@ALS-PG were used as catalysts for hydrolysis of olive oil in 10 h of reaction time. Steapsin lipase in MTMS@ALS-PG and APTMS@ALS-PG shows significantly higher yield because of the restricted leakage of enzymes from the supports when compared to the physically bonded enzyme in ALS-PG. The free steapsin lipase exhibit higher reaction rate but these are unrecoverable from the reaction medium due to its homogeneity.

Esterification of oleic acid with methanol was tested with free and immobilized steapsin lipase at 40 °C. Here, the oleic acid:methanol molar ratio was found to influence strongly the conversion rate. The maximum conversion efficiency was achieved with the acid:alcohol molar ratio 1:6 (Figure S7a) and observed a decline in conversion rate at higher molar ratios. In presence of excess of methanol, probably reverse reaction occurs due to the generation of a large amount of water. While, the presence of n-hexane in the reaction mixture (n-hexane:oleic acid ratio of 5:1 (v/v)), enhanced the methyl oleate yield from 38% to 59%, and shifts the reaction equilibrium from 30 to 12 h (Figure S7b). Probably, the use of n-hexane reduces the inhibitory function of the methanolic substrate in the reaction medium by improving the solvation properties and rate of the reaction. The yields of methyl oleate catalyzed by free steapsin lipase, immobilized steapsin lipase in ALS-PG, MTMS@ALS-PG and APTMS@ALS-PG were 71%, 65%, 56% and 25%, respectively (Figure 8b). The yield of methyl oleate was retained to 59% in MTMS@ALS-PG and 48% in APTMS@ALS-PG after 6 consecutive reuses. However, only 11% of initial conversion could be retained by free steapsin lipase and is completely lost for ALS-PG after 6 cycles (Figure S7c).

The third reaction chosen for testing the catalytic efficiency was the transesterification reaction mainly involved in biodiesel production. Formation and quantification of biodiesel during the transesterification reaction was done by $^1$H NMR. The appearance of a peak at $\delta$ 3.6 due to the formation of −OCH$_3$ supports the formation of biodiesel (Figure 9). The yields of fatty acid methyl esters (FAMEs) that were catalyzed by free, immobilized steapsin lipase in MTMS@ALS-PG and APTMS@ALS-PG were 65%, 53% and 48% and declined to 11%, 38% and 31% after using 6 cycles (Figure S8). The immobilized steapsin lipase showed reasonably higher stability against mechanical or chemical inactivation compared to free one during the chemical reaction and enzyme recovery steps. These results imply the significant potential of immobilized enzyme on hierarchically porous ceramic support for practical applications.

**CONCLUSIONS**

We have demonstrated the synthesis of hierarchically porous alumino-siloxane aerogel microbeads (ALS-PG) via biotemplating method using pollen grains of Hibiscus rosa-sinensis as the sacrificial template. This technique produced aerogel framework with all levels of macro- and mesoscale pore features. The funnel-shaped macrochannels rooted between mesoporous gel framework produced an exciting hierarchical structural morphology and porosity. A high BET/Langmuir surface area (497/664 m$^2$ g$^{-1}$) and total pore volume (1.6788 cm$^3$ g$^{-1}$) with mesopores centered at ca. 3.9, 8.7, 26.6 nm was obtained, when compared to its nontemplated and aerogel counterpart. Steapsin lipase was “comfortably” hosted by modulating the chemical microenvironment via successful functionalization of the support. The study demonstrates that the immobilized steapsin lipase (i) enhanced the protein loading ability (ca. 70 mg g$^{-1}$ support) via hydrophobic and electrostatic interactions in MTMS@ALS-PG and APTMS@ALS-PG, respectively (ii) improved the organic solvent tolerance ability; in particular, nonpolar solvents (iii) showed a better resistance to thermal inactivation @ 45 and 50 °C (iv) retained 80% activity under vigorous mechanical shaking (v) retained 60–70% storage ability @ 30 °C for a period of 30 days (vi) retained more than 50% of their initial activities after 10 times reuse and (vii)
allowed simple recovery and separation of monolithic microbeads from the product, when compared to its free state. Considering the catalytic performances of immobilized steasin lipase to catalyze hydrolytic, esterification and transesterification reactions, it can be a milestone for enzyme-based heterogeneous catalysis. Thus, our study demonstrates an exciting approach of using cost-efficient natural templates to prepare hierarchically porous structures, which can become competitive supports for biological and technological applications.

**ASSOCIATED CONTENT**

2 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.6b00821.

Additional experimental details, figures and tables associated with (i) TGA/DTA, (ii) N$_2$ adsorption analysis, (iii) parameters associated with steasin lipase immobilization and (iv) biocatalytic experiments (PDF).

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Notes

The authors declare no competing financial interest.

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