Research Article Adv. Mat. Lett. 2014, **5**(1), 17-23 ADVANCED MATERIALS Letters

www.amlett.com, www.amlett.org, DOI: 10.5185/amlett.2013.7513 Published online by the VBRI press in 2014

Diastase α-amylase immobilization on sol-gel derived guar gum-gelatin-silica nanohybrid

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Received: 13 July 2013, Revised: 21 August 2013 and Accepted: 01 September 2013

ABSTRACT

In the present communication we report on diastase alpha amylase immobilization at guar gum-silica nanohybrid material (H5). The immobilized amylase (H5-Amyl) showed significantly higher bioactivity (21.62 U mg^{-1}) as compared to free amylase (15.59 U mg^{-1}) in solution at pH 5 and temperature 40 °C. The kinetic parameters of the free (K_m = 10.66 mg L^{-1}; V_{max} = 1.36 μmolemL^{-1}.min^{-1}) and the immobilized enzyme (K_m = 6.11 mg mL^{-1}; V_{max} = 1.45 μmolemL^{-1}.min^{-1}) revealed that the immobilization has increased the overall catalytic property of the enzyme. The immobilized enzyme on recycling could show 87 % of initial activity even in the sixth cycle. Since immobilization did not hamper the enzymatic reaction rate, the biocatalyst may be suitably exploited in food and pharmaceutical industries. Copyright © 2014 VBRI press.

Keywords: Guar gum; gelatin; tetramethoxysilane; sol-gel; amylase; immobilization.

Vandana Singh is Associate Professor of Chemistry at Department of Chemistry, University of Allahabad, India. Her research focuses on the polysaccharides and polysaccharide biotechnology. She could evolve greener methods for polysaccharide transformations such as methylation, hydrolysis and grafting. Currently she is involved in the development of polysaccharide based silica biocomposite for water remediation and enzyme immobilization. She has edited one book and authored over eleven book chapters in books of international repute. She has published seventy three (73) research papers in international journals which include one review article in Progress in Polymer Science. Her publications have attracted >1200 citations with an h-index of 22. She is a fellow of many professional societies in India and abroad.

Devendra Singh is pursuing doctorate degree at University of Allahabad, India. He has obtained a first class postgraduate degree in Chemistry from University of Allahabad. At present he is working as Junior Research fellow in a Council and Scientific & Industrial Research, India funded project. His present work is on the biomimetic synthesis of silica hybrid matrices for enzyme immobilization. He has published one research paper in journal "Process Biochemistry" and presented two papers in conferences.

Introduction

α-Amylase is widely used in food industry such as milling and baking **[1]**. Its immobilization allows controlled engineering design, option for both batch and continuous process and rapid termination of reactions **[2]**. The storage and handling of the enzyme can be simplified by careful selection of the immobilization matrix. Immobilization can also improve the enzyme activity and stability in comparison to free enzyme in solution. Several carrier or support materials are known for enzyme immobilization, which includes natural as well as manmade materials **[3, 4]**. The design of improved immobilized enzymes has always been a challenge for reaping maximum benefit out of any biocatalytic transformation. The simplest method for enzyme immobilization is the nonspecific adsorption involving mainly physical adsorption or ionic binding. Physical adsorption involves hydrogen bonding, van der Waals forces, or hydrophobic interactions for enzyme attachment to the carrier matrix, while in ionic bonding salt linkages allow reversible surface interactions between enzyme and support material. Physical adsorption of enzyme is easy to perform process which normally preserves the catalytic activity of an enzyme nevertheless the adsorption can be easily reversed by changing the conditions. Even being economically attractive, adsorbed enzymes may suffer from the problem of enzyme leakage **[5]**. To minimize the leakage problem, several support matrices have been designed and processed from time to time. The most studied amongst these are the ordered porous materials e.g. mesoporous silicas. Nowadays a variety of materials are available for amylase

immobilization such as periodic mesoporous organosilicas, metal-organic frameworks or magnetic porous materials **[6]**. Poly(divinylbenzene)-1-vinylimidazole)-Cu^{+2} magnetic metal-chelate has been recently used as affinity sorbent for alpha amylase **[7]**. Dye conjugated magnetic beads are also known for amylase immobilization **[8]**.

In general porous metal oxides such as silica **[9]** and zirconia are attractive materials for amylase immobilization **[10]**. Immobilization of α-amylase to hybrid iron(III) hydrous oxide nanostructures has been recently reported **[11]**. Mimicking living organisms such as diatoms, many silica composites have been designed **[12]** specially the dual templated silica hybrids are of recent research interest. Their syntheses involve concomitant templation of silica using proteins and polysaccharides. Silver nanoparticle incorporated gelatin-gum acacia nanocompositional hybrid material **[13]** has been synthesized and successfully used for immobilizing alpha amylase. In another recent study we have used guar gum (polysaccharide component) with gelatin (protein component) for the dual templatation of tetramethoxysilane to obtain an efficient immobilization support for glucose oxidase **[14].** Guar gum is a galactomannan polysaccharide having $\beta(1\rightarrow4)$-linked-D-mannopyranosyl backbone with glycosidically linked α-D-galactose units at the 6-position of every second mannose unit **[15]**.

In the present study, the guar gum-gelatin dual templated silica hybrid has been evaluated for amylase immobilization because amylase has high biotechnological importance in food, pharmaceutical, textile and paper industries. Microbial amylases have now completely eliminated the use of chemicals for hydrolyzing starch in starch processing industries **[17].** Its immobilization can allow the reuse or continuous use of the biocatalyst for the extended period of time **[18].** This study is important for understanding the material's bioactivity and selectivity among the different classes of enzymes. Diastase alpha amylase is a multimeric metalloenzyme, while glucose oxidase is dimeric in nature. Amylase catalyzes the hydrolysis of α-1,4 glucan linkages in starch **[16]**, while glucose oxidase catalyzes the oxidation of D-glucose to gluconic acid **[15]**. It is expected that on immobilization these two enzymes may not show similar behavior.

This is the first report of this type where guar gum has been used as dual template component for templating silica nanohybrid to obtain amylase carrier support material.

Experimental

Materials and methods

Tetramethoxysilane (98% TMOS; Merck, Germany) was used as silica precursor. Guar gum (Merck) was used as supplied. Analytical grades of fungal diastase amylase (Merck, Germany), 3, 5-dinitrosalicylic acid, NaOH, HCl and phosphate salts (Merck, India) were used. Soluble starch (Merck Germany (M_v = 4451 Da) was used. Gelatin, (Qualigens) was used.

Instrumentation

X-ray diffraction (Cu/Kα-source) of the powdered samples was carried out on XRD Pananalytical X-Pert Pro X-ray powder diffractometer. IR was done by forming KBr pellets through Fourier transform infrared (FTIR) spectrophotometer, JASCO FTIR, within the spectral range of 400–4000 cm^{-1} and resolution 4 $cm.^{-1}$ SEM analyses were done on JEOL model JXA8100 machine. Surface area of the hybrid gels was determined by WT Classic BET Surface Area Analyzer WAKO, India at 77 K, for which prior to gas adsorption, all the samples were degassed for 4 h at 533 K. Spectrophotometric measurements were made using Cyberlab UV-vis double beam spectrophotometer (model UV-100) using quartz cuvets. Temperature treatment of the hybrids was carried out in N_2 using electric muffle furnace (Metrex Scientific Instruments (P) Ltd., New Delhi). After calcination, the materials were left inside the muffle furnace for cooling to room temperature.

Enzyme stock solution

The diastase alpha amylase (Amyl) stock solution was prepared by dissolving 100 mg of solid diastase alpha amylase in 250 mL of distilled deionized water.

Dialysis of enzyme

Enzyme solution (5 mL of 400 μg mL^{-1}) contained in dialysis bag (1 inch width) was hanged in 2 L of 0.02 M phosphate buffer solution for 8 h. This process was repeated thrice after which the bag was dipped in 0.02 M sugar solution bed for 4 h for the dialysis.

Table1. Synthesis optimization of the hybrid: TMOS (1.5) and MeOH (1.5 mL) & H_2O (15 mL).

S.No.	Hybrid	pH	GG (mg)	Gelatin (mg)	Gel Time (h)	Yield (g)	Caln Temp (ºC)	Activity (Umg^{-1})
1	C_a	4	100	10	7	720		12.93
2	C_b	4.5	100	10	7	840		14.61
3	C_c	5	100	10	7	800		16.37
4	C_d	5.5	100	10	7	785		18.47
5	C_e	6	100	10	7	842		1.90
6	C_f	6.5	100	10	7	790		4.32
7	C_g	8	100	10	7	835		3.59
8	C_{da}	5.5	100	5	7	763		9.32
9	C_{db}	5.5	100	7.5	7	770		19.35 (H1)
10	C_{dc}	5.5	100	10	7	785		18.11
11	C_{dd}	5.5	100	12.5	7	792		15.04
12	C_{de}	5.5	100	15	7	798		12.24
13	C_{df}	5.5	100	20	7	820		8.49
14	C_{de}	5.5	100	30	7	835		13.05
15	H2	5.5	100	7.5	7	770	200	9.24
16	H4	5.5	100	7.5	7	770	400	12.811
17	H5	5.5	100	7.5	7	770	500	21.62
18	H6	5.5	100	7.5	7	770	600	21.03
19	H7	5.5	100	7.5	7	770	700	19.68
20	CS	5.5	---	---	6	297	500	11.98
21.	F_{ENZ}	-	-	-	-	-	-	15.59

Activity measurement: 100 mg H5-Enz, temperature = 40 ^{0}C, incubation time = 10 min, RPM=200, pH= 5, at 540 nm as described in 2.6.2.

Material synthesis

The optimization of control hybrids (singly templated) is described elsewhere **[15],** which was necessary to optimize the gelling time of the dual templated hybrids. Based on the process parameters required for the synthesis of singly templated hybrids, gum-gelatin dual templated silica hybrids (C_a to C_g) were synthesized using 100 mg of guar gum in 15 mL deionized water having a preadjusted pH (pH was adjusted using the required amount of acid or alkali) **(Table 1)**. To this solution gelatin (10 mg), TMOS (1.5 mL) and MeOH (1.5 mL) were added and the mixture was stirred for 7 h on a magnetic stirrer at room temperature (30 ºC). The gel was washed with water and

dried under reduced pressure. At optimum pH (5.5), the amount of gelatin was again optimized using the synthetic conditions of C_d, the optimum C series hybrid. The hybrid hydrogels thus obtained were washed well and dried to obtain hybrid samples C_{da} to C_{de}. C_{dc} being the optimum performance sample, was calcined at different temperatures from 200 to 700 °C (2 h at each temperature) for obtaining the hybrid xerogels (H2-H7) **(Table 1)**. In a preliminary investigation, these hybrids were evaluated for alpha amylase immobilization as described in the following sections and their bioactivities were assayed.

Immobilization of enzymes

0.1 mL of diastase (0.4 mg/mL) aqueous solution was adsorbed at 100 mg of the presynthesized hybrid sample (H5) and left for 2 h at 40 ºC. The enzyme impregnated hybrid was dried in vacuum (at 40 ºC) after a thorough wash with de-ionized distilled water. The immobilization of the enzyme was done at a definite pH (pH 5.5) that was adjusted using phosphate buffer (0.02 M NaH_2PO_4, 0.02 M Na_2HPO_4, and phosphoric acid). Our trial experiments revealed that pH 5.5 was most suitable for optimum enzyme loading (results not shown).

Activity of the immobilized amylase

The activity of diastase alpha amylase was monitored using 3,5-dinitrosalicyclic acid (DNS) method **[19]** which involves the monitoring of the released reducing sugar. The activity was assayed for 1 mL reaction mixture containing soluble starch (0.5 mL of 1% w/v in 0.02 M phosphate buffer), free diastase alpha amylase (0.1 mL of 0.4 mg/mL (w/v) ≈ 40 µg) and distilled H_2O (0.4 mL) at pH 5 after 10 min incubation at 40 ºC. The hydrolysis of soluble starch was also monitored under identical conditions using the immobilized enzyme (100 mg hybrid containing 40 µg of diastase) with 0.5 mL of H_2O, and 0.5 mL of 1% (w/v) starch solution in 0.02 M phosphate buffer at pH 5 after 10 min incubation at 40 ºC. The enzymatic reaction was interrupted by adding 1 mL 3,5-dinitrosalicylic acid as coupling reagent, followed by heating in boiling water for 4 min. After adding 10 mL of cold water, the reaction mixture was cooled and centrifuged and the optical density of the centrifugate containing the brown reduction product was determined spectrophotometrically at λ_{max} 540 nm. A blank experiment was also performed under the identical conditions but in absence of enzyme. Diastase alpha amylase activity was expressed in terms of micromoles of the reducing sugar. The amount of reducing sugar released during the reaction was estimated with the help of maltose standard curve. Enzyme activity was determined as described above. One unit of α-amylase activity is defined as the amount of enzyme, which produces reducing ends equal to 1.0 µmol maltose in 1 min at 40 ºC and pH 5. Enzyme (40 µg) impregnated H5 (H5-Enz) was used for optimizing the soluble starch hydrolysis, where the weight of the enzyme refers to the weight of solid enzyme.

The hydrolysis of starch was done using pre-dialyzed free diastase alpha amylase and H5-Amyl at different substrate concentrations, temperatures and pH values.

To study the effect of pH on the enzymatic hydrolysis of soluble starch, 0.1 mL of 0.4 mg mL^{-1} enzyme was added to 0.5 mL of 1% (w/v) starch solution that had been preadjusted to different pH values (pH 4 to 9) with the help of phosphate buffer [**20**]. After the addition of 0.5 mL H_2O, the reaction mixture was incubated at 40 ºC for 10 min. To study the pH effect on the immobilized enzyme hydrolysis, a known weight of H5-Amyl was added to 0.5 mL of 1% (w/v) starch solution that had been pre adjusted at different pH values [**20**].

To study the effect of temperature on the enzymatic hydrolysis, 0.5 mL of 1% (w/v) starch solution was hydrolyzed at pH 5 with 0.1 mL of 0.4 mg/L free enzyme solution at different temperatures ranging from 25 ºC to 60 ºC after 10 min incubation in a temperature controlled incubator shaker set at 200 rpm. For the immobilized enzyme, a known weight of H5-Enz was incubated for 10 min at different temperatures ranging from 25 ºC to 60 ºC with 0.5 mL of 1% (w/v) starch and 0.5 mL of H_2O at pH 5.

After the first cycle, H5-Enz was separated from the reaction mixture by centrifugation and was stored for recycling after a thorough wash with distilled water. Recycling studies were done for exactly the same duration and under the conditions used for the first cycle with 100 mg of recycled H5-Amyl. For the kinetic study, concentration of starch was changed from 0.25 to 2.0 % (w/v) at pH 5, while keeping the other conditions same as described above.

For evaluating the shelf life, H5-Amyl was stored at room temperature (~35 ºC) as a dry solid for 25 days while soluble amylase was stored in phosphate buffer and their bioactivities were monitored as described above. All the experiments were performed in triplicate and the results reported are the average of three readings.

The % loading of the enzyme was calculated as below.

$$\%\ \text{Enzyme loading} = \frac{U_{total} - U_{remain}}{U_{total}} * 100 \quad (1)$$

Where, U_{total} is the activity of total free enzyme that was loaded on the carrier support, and U_{remain} is the activity of amylase in filtrate.

Stability of the enzyme

Free and the immobilized enzymes were monitored for their room temperature stability. The free enzyme was stored in pH 5 phosphate buffer while the immobilized enzyme was stored as dry solid at room temperature (30 °C) for 15 days **[20]**. The activities of the stored enzyme samples were monitored as described above.

Results and discussion

The optimum sample of guar gum-gelatin-silica hybrid was obtained using 100 mg of guar gum, 7.5 mg of gelatin, 15 mL H_2O, 1.5 mL TMOS, 1.5 mL MeOH at pH 5.5 **(Table 1).** The parameters involved in a hybrid synthesis can dictate the final characteristics and the microstructure of the polysilicate material. The hybrid synthesis involves successive hydrolysis and condensation steps which may be influenced by solvent, catalytic & steric effects besides the templating environment. At pH <4, the gelation time was much prolonged so the hybrids were synthesized in the pH range of pH 4 to pH 8. As the isoelectric point and

point of zero charge for silica range between pH 1-3, at pH >4, deprotonated silanols are involved in condensation. In the present study, the gelling time for the whole pH range was ~7 h.

The characterization of the optimum activity material has been described elsewhere **[15]**. The silica incorporation in the hybrid was confirmed by silica related peaks below 1250 cm.$^{-1}$ Infra red spectrum of H5 showed bands characteristic to silica matrix (463 cm^{-1}, 797 cm^{-1}, 1087 cm^{-1}) as well as guar gum 3464 cm^{-1} (SiO-H stretching overlapped with Guar gum hydroxyl), 2937 cm^{-1} (CH_2 stretching), 2890 cm^{-1} (CH stretching) and O-H bending (1639 cm^{-1}). There was no significant difference in the FTIR spectrum of H5 and H5-Amyl **(Fig. 1)**. The immobilization may be due to physical forces as after Amyl immobilization combined peak of SiO-H and guar gum hydroxyl have been shifted significantly (to 3452 cm^{-1}), indicating their involvement in the enzyme impregnation. A marginal shift in O-H bending peak (1644 cm^{-1}) was also observed. Other silica related peaks in H5-Amyl are seen at 1086 cm,$^{-1}$ 799 and 475 cm^{-1}. The H5 is an amorphous material having surface area of 35.76 m^2/g **[15]**. H5-Amyl showed higher surface area (42.08 m^2 g^{-1}) as now enzyme surface is also included in the total surface area.

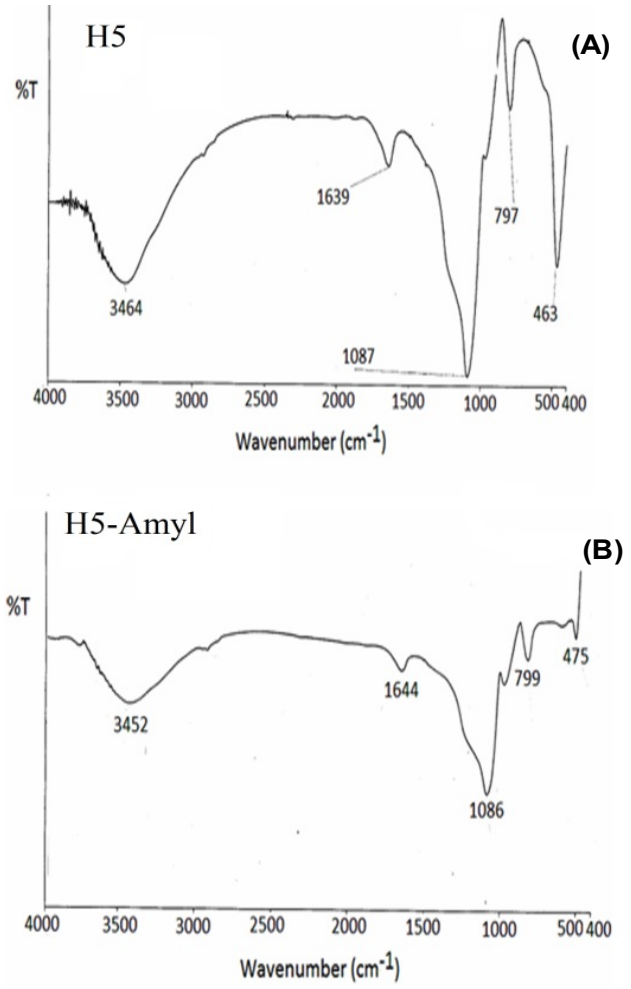

Fig. 1. FTIR spectra of **(A)** H5 and **(B)** H5-Amyl.

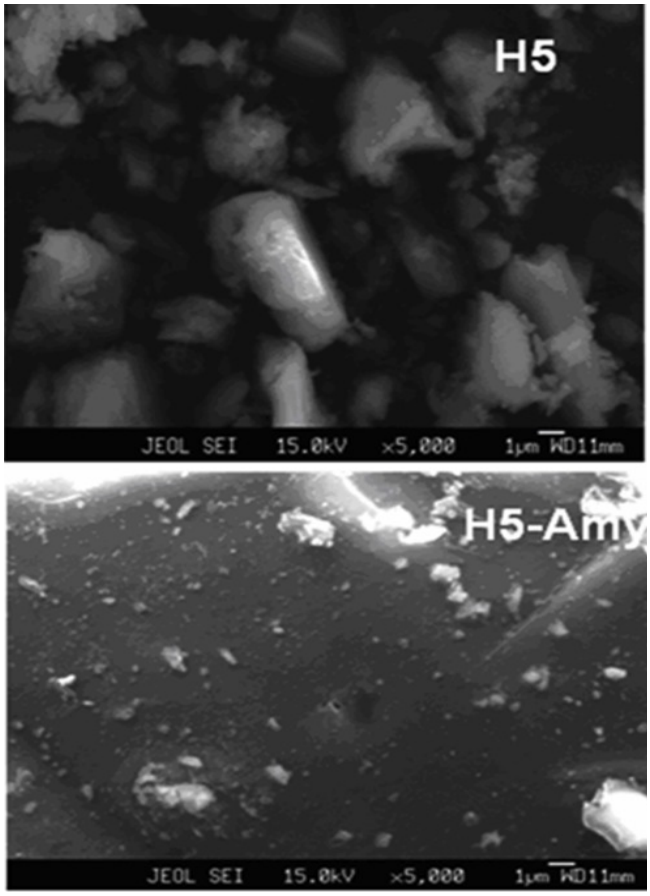

Fig. 2. SEM pictures of H5 and H5-Amyl.

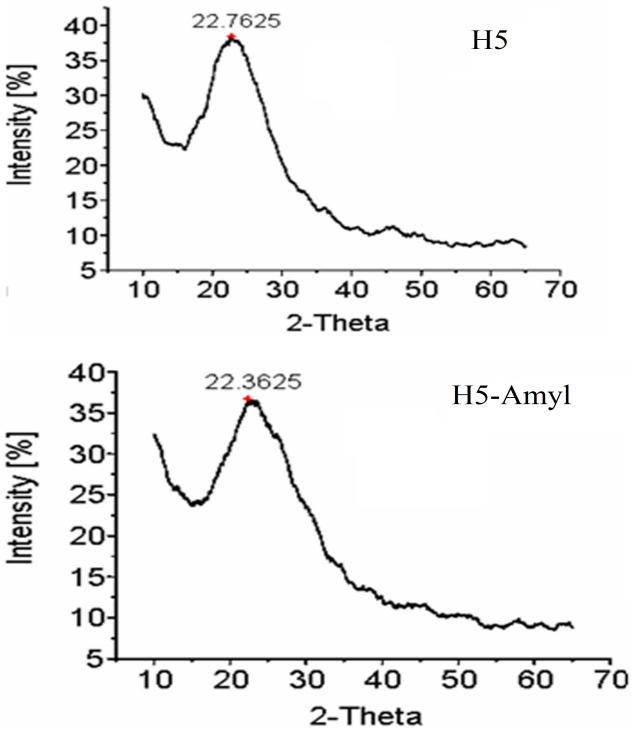

Fig. 3. XRD patterns of H5 and H5-Amyl.

SEM studies **(Fig. 2)** revealed that the H5 has smooth surfaced scattered bulk particles, which show totally different surface topology on amylase immobilization. The

immobilization of enzyme did not change the diffraction pattern of H5 **(Fig. 3)**, both H5 and H5-Amyl have same diffraction angle centres (2θ 22°), thus the enzyme impregnation did not change the amorphous nature of the hybrid.

Enzyme immobilization study

The synthesized materials were monitored for their ability to carry α-Amylase. The immobilized enzymes thus obtained were screened for their bioactivities. The amount of the hybrid and enzyme to be loaded were chosen depending upon the results of our trial experiments for which hybrid to enzyme ratio was varied and the bioactivities of the immobilized enzymes thus obtained were monitored for the optimum results. (Preliminary results are not shown). Hydrolysis of the soluble starch has been optimized using H5-Amyl and the kinetic parameters were derived. The percentage loading of Amyl at the hybrid sample (H5) was calculated (c.f. equation-1) to be 84 %.

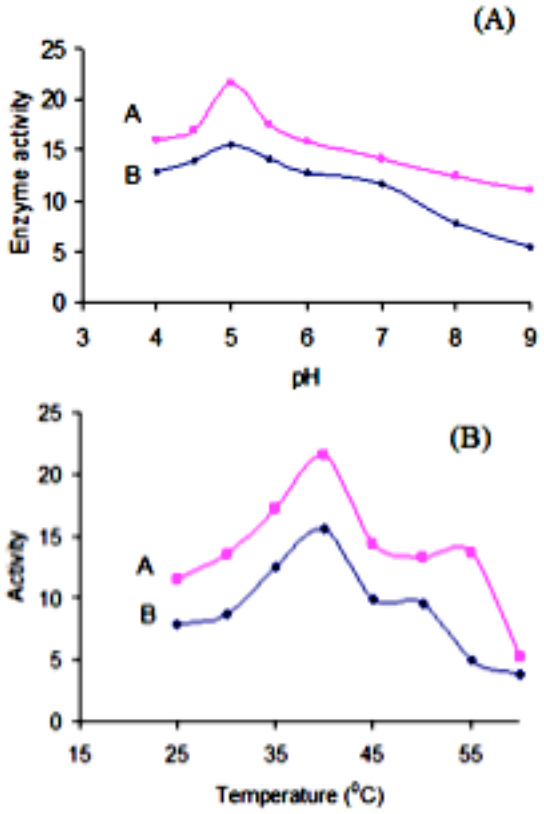

Fig. 4. (A) Enzyme bioactivity at different pH; A. immobilized enzyme, B. free enzyme; and **(B)** Enzyme bioactivity at different temperatures; A. immobilized enzyme, B. free enzyme.

Enzyme immobilization

100 mg of H5 was used for immobilizing Amyl. About 84 % of the loaded amylase was adsorbed at H5.

Effect of pH on enzyme bioactivity

The pH optimum of the studied enzymatic reactions was determined by varying the pH of the assay reaction mixtures using the phosphate buffers **(Fig. 4(A))**. The residual enzyme activity was determined as described earlier. The bioactivities of both free and the immobilized enzymes were pH sensitive but the immobilization did not change the pH-response of the enzyme **(Fig. 4(A))**.

The enzyme activity (for free and immobilized) was optimum at pH 5. The strength of the electrostatic interaction between the enzyme and the silica support is very important in deciding the overall activity of the enzyme. As pH was increased, the activity of enzyme (in free as well as in the immobilized states) declined. The activity of immobilized enzyme is higher as compared to free enzyme in the studied pH range.

Effect of temperature on enzyme bioactivity

The temperature effect on the soluble starch hydrolysis was studied using H5 and H5-Amyl **(Fig. (4B))**. Both free and immobilized enzyme (H5-Amyl) showed identical trend on increasing the temperature of the biocatalytic reaction. Sharp decrease in the enzyme activity with increase in temperature above 55 ºC indicated some denaturation of the enzyme.

Hydrolysis and kinetic parameters

The kinetic parameters for free and the immobilized enzymes have been determined at pH 5 at 40 ºC. The relation between substrate concentration and rate of enzymatic reaction can be described by Michaelis-Menten (MM) equation, which can be represented into linear form as Lineweaver-Burk equation for computation of V_{max} (the rate of reaction in mg $mL^{-1}.min^{-1}$), and K_m (Michaelis constant in mg mL^{-1}) values. K_m of an immobilized enzyme may differ from free enzyme due to steric effects **[21]**, diffusion limitations **[22]** and ionic strength **[23]**. Kinetic parameters, the Michaelis constant K_m and the maximum enzyme activity V_{max} has been determined using the Lineweaver plots **(Fig. 5)**.

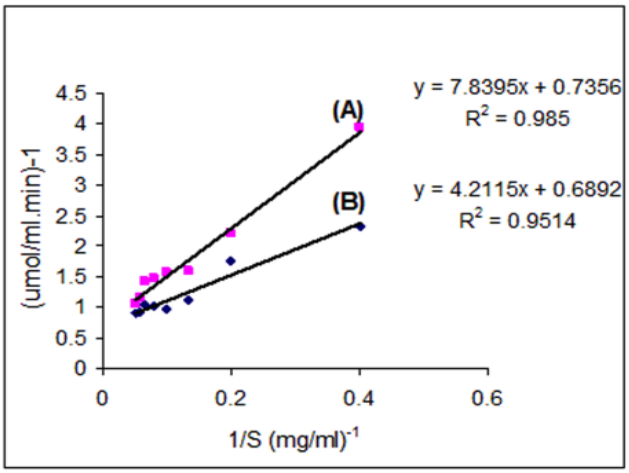

Fig. 5. Lineweaver Burke plots of (A) free amylase (B) H5-Amyl.

The kinetic parameters of the free (K_m = 10.66 mg mL^{-1}; V_{max} = 1.36 µmole $mL^{-1}.min^{-1}$) and immobilized enzyme (K_m = 6.11 mg L^{-1}; V_{max} = 1.45 µmole $mL^{-1}.min^{-1}$) revealed a significant decline (1.8 fold) in K_m value that indicates an increase in the enzyme substrate affinity. Thus the

immobilization leads to a favorable change in the Amyl conformation or decrease in enzyme inhibition. V_{max} values indicate that the maximum rate of amylase reaction increases upon the immobilization. Under the optimized conditions the immobilized amylase (H5-Amyl) had higher bioactivity (21.62) than free amylase (15.59 U mg^{-1}) though this increase was less prominent than what we observed for H5 immobilized glucose oxidase (H5-GOX) activity where the immobilization had nearly doubled **[15]** the bioactivity. This indicated that the H5 is more efficient in immobilizing dimeric glucose oxidase in comparison to multimeric enzyme, diastase alpha amylase. Another importance difference is that immobilized amylase has higher value of V_{max} than free amylase while for immobilized glucose oxidase there was slight decrease in V_{max} value. It may be due to the specific structure of glucose oxidase, where active site (where glucose binds) is present in deep pocket, which becomes more difficult to approach upon the immobilization **[24]**.

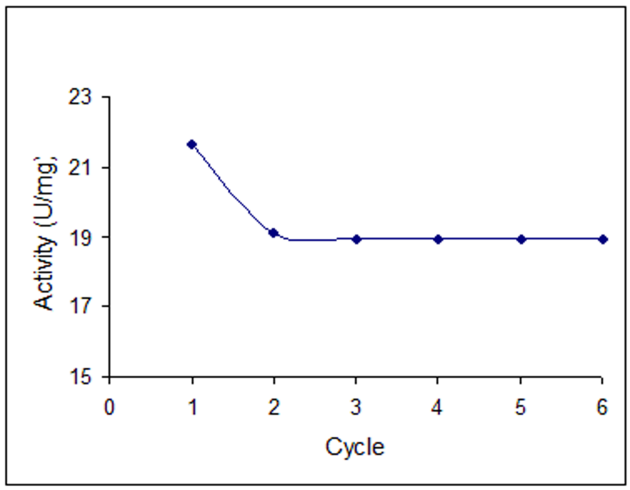

Fig. 6. Recycling of H5-Amyl.

Recycling and storage

The immobilized enzyme (H5-Amyl) was recycled for six cycles. There was ~11 % loss in the bioactivity of H5-Amyl after the first cycle; however in subsequent cycles there was no loss in the bioactivity of the immobilized enzyme. It appears that the weakly adsorbed enzyme is lost in the initial cycles. The immobilized Amyl retained 87% of its initial activity even in the sixth cycle **(Fig. 6)**.

The free Amyl was stored in phosphate buffer of pH 5, while the immobilized enzyme was stored as dry solids for 15 days at room temperature. There was a marginal loss in the bioactivity of the enzyme (free and immobilized) on storage. After 15 days storage in the said environment the bioactivities of free and the immobilized enzymes were 20.44 Umg^{-1} and 14.52 Umg^{-1} respectively. The immobilization did not result much advantage in terms of storage stability but the storage is now easier as it can be stored at dry solid at room temperature.

Conclusion

Guar gum-gelatin dual templated hybrid hydrogel on calcination furnished porous bio-inspired silica hybrid that efficiently immobilized diastase α-amylase. The immobilization did not affect the optimum pH and temperature for the biocatalytic reaction. The immobilization enhanced the enzyme substrate affinity and the enzymatic reaction rate. The substrate is suitable for repeated use of the alpha amylase in biotechnological applications as in soluble state the enzyme is prone to aggregation and interaction with hydrophobic interfaces.

Acknowledgements

Authors thank Council of Scientific & Industrial Research (C.S.I.R.), New Delhi, India for financial support to carryout the present work and to Centre for Mineralogy and petrology for SEM and XRD facility. IR is acknowledged to Banaras Hindu Univeristy, Varanasi, India

Reference

1. Contesini, F.J.; Figueira, J. de A.; Kawaguti, H.Y.; Fernandes, P.C. de B.; Carvalho, P. De. O. *Int. J. Mol. Sci*. **2013**, *14*, 1335-1369. **DOI:** 10.3390/ijms14011335
2. Beatriz, M. B.; Francisco, B.-V. *In Methods in Biotechnology: Immobilization of Enzymes and Cells*, Second Edition, Guisan J. M. (Ed.); Humana Press Inc., Totowa, NJ, **2006**, pp 15-30. **ISBN:** 978-1-58829-290-2
3. Kim, B.C.; Nair, S.; Kim, J.; Kwak, J.H.; Grate, T.W.; Kim, S.H.; Gu, M. B. *Nanotechnology* **2005**, *16*, S382-S388. **DOI:** 10.1088/0957-4484/16/7/011
4. Morais, R.R.; Pascoal, A.M.; Caramori, S.S.; Lopes, F.M.; Kátia F.; Fernandes, K.F. *Enzyme Research*, **2013**, Article ID 803415. **DOI:** 10.1155/2013/803415
5. Sheldon, R.A. *Adv. Synth. Catal.* **2007**, *349*, 1289-1307. **DOI:** 10.1002/adsc.200700082
6. Fried, D.I.; Brieler, F.J.; Fröba, M. *Biocatalysis*, **2013**, **DOI:** 0.1002/cctc.201200640
7. Osman, B; Kara, A; Demirbel, E; Kok, S.; Besirli, N. *Appl. Biochem. Biotechnol.* **2012**, *168*, 279-294. **DOI:** 10.1007/s12010-012-9771-z
8. Tuzmen, N.; Kalburcu, T.; Denizli, A. *J. Mol. Cat. B-Enz*. **2012**,*78*,16-23. **DOI:** molcatb.2012.01.017
9. Soleimani M.; Khani, A.; Najafzadeh, K. *J. Mol. Cat. B: Enzym.*, **2012**,*74*, 1–5. **DOI:** j.molcatb.2011.07.011
10. Reshmi, R.; Sanjay, G.; Sugunan, S.; *Catalysis Commun*. **2007**, *8*, 393-399. **DOI:** 10.1016/j.catcom.2006.07.009
11. Bellino, M. G.; Regazzoni, A. E. *App. Cat. A-Gen.* **2011**, *408*, 73-77. **DOI:** 10.1016/j.apcata.2011.09.008
12. Doumèche, B.; Picard, J.; Larreta-Garde, V. *Biomacromol.* **2007**, *8*, 3613-3618. **DOI:** 10.1021/bm700540p
13. Singh, V.; Ahmed, S. *Int. J. Biol. Macromol.* **2012**, *50*, 353-361. **DOI:** 10.1016/j.ijbiomac.2011.12.017
14. Singh, V.; Singh, D. *Ind. Eng. Chem. Res.* **2013**, under review.
15. Singh, V.; Kumar, P.; Sanghi, R. *Prog. Polym. Sci.* **2012**, *37*, 340–364. **DOI**: 10.1016/j.progpolymsci.2011.07.005
16. Marc, J.E.C.; van der, M.; Veen, B. v. d; Uitdehaag, J.C.M.; Leemhuis, H.; L. Dijkhuizen, L. J. Biotechnol. **2002**, *94*, 137–155. **DOI:** 0 168-1656 (01)00407-217.
17. Das, S. *Intern. J. Pharm. Biosci.,* **2011***, 2, 486-496.* **ISSN:** 0975-6299
18. Guisan, J.M. *In Immobilization of enzymes and cells,* Guisan, J.M (Ed.); Humana Press, Totawa, New Jersey, 2nd edition, 2006, pp 1-15. **DOI:** 10.1007/978-1-59745-053-
19. Hu, R.; Lin, L.; Liu, T.; Ouyang, P.; He, B.; Liu, S. *J. Biobased Mater. Bioenergy*. **2008**, *2*, 156-161. **DOI:** 10.1166/jbmb.2008.306
20. European Pharmacopeia,5.0, Council of Europe (COE)—*European Directorate for the Quality of Medicines (EDQM)*,Chapter 4, Reagents, section 4.1.3., Buffer solutions, **2005**, pp 430. **DOI:** 01/2005:40000.
21. Tischer, W.; Wedekind, F. In *Topics in Current Chemistry*, Fessner, W.D. (Ed.); Springer, Berlin Heidelberg, **1999**, pp. 95–126. **DOI:** 10.1007/3-540-68116-7_4
22. Siso, M.I.G.; Graber, M.; Condoret, J-S.; Combes, D. J. *Chem.Technol. & Biotechnol.* **1990**, *48*, 185-200. **DOI:** 10.1002/jctb.280480208

23. Manelius, R.; Bertoft, E. *J. Cer. Sci.* **1996**, *24*, 139-150.
DOI:10.1006/jcrs.1996.0047,
24. Hecht, H. J.; Kalisz, H.M.; Hendle, J.; Schmid, R.D.; Schomburg, D.
J.Mol. Biol. **1993,** *229*, 153–172
DOI: 10.1006/jmbi.1993.1015

