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Upgrading of biomass monosaccharides by immobilized glucose dehydrogenase and xylose dehydrogenase

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Abstract: Direct upgrading and separation of the monosaccharides from biomass liquors is an overlooked area. In this work we demonstrate enzymatic production of gluconic acid and xylonic acid from glucose and xylose present in pretreated birchwood liquor by glucose dehydrogenase (GDH, EC 1.1.1.118) and xylose dehydrogenase (XDH, EC 1.1.1.175), respectively. The biocatalytic conversions were compared using two different kinds of silica support materials (silica nanoparticles (nanoSiO₂) and porous silica particles with hexagonal pores (SBA silica) for enzyme immobilization. Upon immobilization, both enzymes showed significant improvement in their thermal stability and robustness at alkaline pH and exhibited over 50% activity even at pH 10 and 60°C on both immobilization matrices. When compared to free enzymes at 45°C, GDH immobilized on nanoSiO₂ and SBA silica displayed a 4.5 and 7.25 fold increase in half-life, respectively, whilst XDH immobilized on nanoSiO₂ and SBA showed a 4.7 and 9.5 fold improvement in half-life, respectively. Additionally, after five reaction cycles both nanoSiO₂GDH and nanoSiO₂XDH retained more than 40% activity and GDH and XDH immobilized on SBA silica maintained around 50% of their initial activity resulting in about 1.5-1.6 fold increase in biocatalytic productivity compared to the free enzymes.

Introduction

Bio-based conversion of biomass components by soluble or immobilized enzymes has been presented as a promising and efficient approach for sustainable production of valuable chemical compounds under mild reaction conditions^[1,2]. Use of glucose dehydrogenase (GDH) (EC 1.1.1.118) and xylose dehydrogenase (XDH) (EC 1.1.1.175) is of interest in this regard because these NAD⁺ dependent enzymes catalyze conversion of D-glucose into gluconic acid and D-xylose into xylonic acid, respectively^[3]. Besides transformation of monosaccharides, the enzymatic conversion adds a charge to the products, which can facilitate separation of acid products from a mixed product stream, for instance using membrane technology^[4]. Both gluconic acid and xylonic acid are classified by the US Department of Energy among the top 30 potential high-value

compounds from biomass^[5]. Gluconic acid is a mild organic acid that has multiple applications in the food industry but also in pharmaceuticals synthesis^[6]. Xylonic acid is used as a substrate for synthesis of 1,2,4-butanetriol and 1,2,4-butanetriol trinitrate^[7] and has moreover been projected for various uses in the food, pharmaceutical and agriculture industries^[8]. However, the practical biocatalytic conversion of biomass components using GDH and XDH is limited due to their relatively low stability at extreme pH and temperature conditions.

A possible approach to overcome these limitations and at the same time increase the biocatalytic productivity via maximizing enzyme "reuse" is enzyme immobilization^[4,9]. Enzyme immobilization may moreover reduce the complexity of enzyme separation from the products after reaction^[6,10]. Various techniques and methods of immobilization such as adsorption, covalent binding, entrapment or encapsulation have been described previously^[11-13]. Silica-based materials are frequently used as support materials in enzyme immobilization due to their thermal, chemical and mechanical resistance, good sorption properties, and the presence of many hydroxyl groups that facilitate enzyme binding^[14-16]. Moreover, silica-based materials are easy to obtain and relatively cheap, a feature of particular significance in biomass valorization processes. Nonetheless, data related to immobilization of glucose dehydrogenase and xylose dehydrogenase on silica are limited.

As data about immobilization of glucose dehydrogenase and xylose dehydrogenase are limited, a simple protocol is required to use efficient and stable support materials for immobilization of GDH and XDH which additionally facilitating further separation of products of enzymatic conversion. Thus, in the present study, we examine immobilization, characterization and comparison of GDH and XDH on two types of silica support materials – silica nanoparticles and mesoporous silica with hexagonally ordered pores. As part of the study, we also investigate the kinetics of the enzymatic conversions of monosaccharides, and examine the effect of various pH and temperature conditions on free and immobilized GDH and XDH and evaluate the influence of these parameters on the enzyme stability. The practical application of the biocatalytic systems is validated by applying them for conversion of glucose and xylose present in authentic pretreated birch wood biomass liquors.

Results and Discussion

GDH and XDH immobilization

In this study, silica nanopowder of particle size in the range of 10–20 nm and pore diameters of around 2 nm size (nanoSiO₂) and mesoporous SBA 15 silica (<150 µm particle size) with hexagonal pore morphology and pore size up to 20 nm (SBA15) were used. It should be emphasized that type of the support material is known to affect place of enzyme binding. According to the previously published articles, using silica nanoparticles,

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GDH and XDH are expected to be immobilized on the particle surfaces, while in case of SBA silica, biomolecules are presumably bound mainly inside the SBA 15 silica pores, as it is schematically presented in Fig. 1^[14,15].

Figure 1. Schematic presentation of immobilization of glucose dehydrogenase and/or xylose dehydrogenase: (a) on silica nanoparticles and (b) in pores of hexagonal mesoporous silica.

These expectations were confirmed by the images from transmission electron microscopy and changes of the porous structure parameters of silica-based materials after GDH and XDH immobilization (Fig. 2 and Table 1). After immobilization of both enzymes surface area of the nanoSiO₂ decreased around two times and reached about 120 m²/g as its pore size was

unaltered after enzyme binding. Surface area of SBA 15 silica was intact after immobilization, meanwhile significant changes were noticed in pore size and pore volume indicating GDH and XDH immobilization into the pores of the hexagonal silica.

Table 1. Porous structure parameters of nanoSiO₂ silica and hexagonal mesoporous silica SBA 15 before and after immobilization of glucose dehydrogenase or xylose dehydrogenase.

Sample name	BET surface area (m ² /g)	Pore volume (cm ³ /g)	Pore size (nm)
nanoSiO ₂	219.6	0.098	2.048
nanoSiO ₂ GDH	118.3	0.087	2.046
nanoSiO ₂ XDH	121.6	0.091	2.047
SBA15	579.6	0.868	19.211
SBA15GDH	567.2	0.476	14.754
SBA15XDH	570.2	0.513	15.634

Figure 2. TEM images of the: (a) SBA 15 silica and (b) nanoSiO₂ silica before enzyme immobilization; (c) and (e) after immobilization of glucose dehydrogenase; (d) and (f) after immobilization of xylose dehydrogenase.

Table 2. Immobilization yield and amount of immobilized enzyme in different biocatalytic systems. Specific activity and activity retention of free and immobilized GDH and XDH.

Analyzed parameter	freeGDH	nanoSiO ₂ GDH	SBA15GDH	freeXDH	nanoSiO ₂ XDH	SBA15XDH
Amount of immobilized enzyme (mg/g);*(U/g)	-	2.8±0.1' 560±19*	2.5±0.1' 500±14*	-	534±16*	450±12*
Immobilization yield (%)	-	94±3.5	82±2.8	-	89±3.0	75±2.7
Specific activity (U/mg)	42.2±0.9	30.5±0.8	27.3±1.1	46.8±1.7 U/ml	29.0±0.7	24.6±0.8

The different ways of immobilization were thus anticipated to produce different amounts of immobilized enzymes, highest on the nanoparticles due to their high surface area: 2.8 mg (560 U) of GDH and 534 U of XDH were immobilized on 1 g of nanoSiO₂, which was about 10–15% higher compared with SBA 15 (Table 2). Immobilization yield also followed this trend: when nanoSiO₂ was used, immobilization yield for GDH and XDH reached 94 and 82%, whereas for SBA 15 it was 89 and 75%, respectively. Hence, irrespective of the support material, a higher quantity of the immobilized enzyme was noticed in case of glucose dehydrogenase. Greater amounts of immobilized GDH, as compared to XDH, are probably related to the three-dimensional structure and amino acids composition of this enzyme^[17]. A possible explanation for this is that enzymes molecules possessing more accessible amino acids in their structure, like arginine, asparagine, glutamic acid or lysine are more effectively immobilized, as it was previously reported for glucose-6-phosphate dehydrogenase^[18]. Nevertheless, both enzymes are linked to the silica by adsorption immobilization, by creation of electrostatic interactions and hydrogen bonds between mainly amino (–NH₂), hydroxyl (–OH) and carbonyl (–COOH) groups of the enzymes and hydroxyl groups of the silica-based support.

The specific activity of GDH and XDH was 42.2 U/mg and 46.8 U/ml, respectively, indicating that both enzymes showed similar catalytic activity in model reaction. The specific activity of the immobilized enzymes was lower and reached 30.5 and 27.3 U/mg of the enzyme, respectively, for nanoSiO₂GDH and SBA15GDH, which corresponded to an activity retention of 72.3 and 64.7%, respectively. For nanoSiO₂XDH and SBA15XDH, these values were slightly lower and reached 29.0 and 24.6 U/mg, respectively. Also noticed was a lower activity retention of 61.8% for nanoSiO₂XDH and 52.6% for SBA15XDH. The lower values of specific activity and activity retention noticed for enzymes immobilized onto SBA 15 compared with nanoSiO₂, might be explained by two factors: (i) lower amount of immobilized biocatalysts and (ii) hindered accessibility of the enzyme active sites for the substrates molecules due to immobilization of enzymes mainly into the pores of the support. Nevertheless, lower activity of immobilized XDH compared with GDH is probably related to the larger conformational changes of 3-D biomolecule structure that occur upon immobilization, as reported earlier by Li et al.^[19].

There is no available literature about efficient immobilization of XDH, and though there are some reports about immobilization of GDH, results presented here go further than previously published data. For example, Baron et al. used controlled pore silica with average pore size of 500 Å, as a support for *Bacillus*

megaterium glucose dehydrogenase and immobilized less than 0.5 mg of enzyme per 1 g of the support^[20], whereas here we report a loading capacity of silica nanoparticles of 2.8 mg/g, probably due to higher density of hydroxyl groups on the surface of nanoSiO₂.

Effect of temperature and pH on activity and stability of free and immobilized GDH

Free GDH as well as both of the GDH-based, immobilized systems exhibited the highest activity at 45°C (Fig. 3a). At temperatures below optimum, free GDH was characterized by slightly higher, but significantly different, activity than the immobilized enzyme. But at higher temperatures (45–60°C), immobilized GDH showed better activity than the free biocatalyst. This is particularly noticeable in the case of the mesoporous SBA 15 silica support and GDH immobilized on this material showed higher residual activity (53%) than free enzyme (29%) even at a temperature of 60°C. GDH is known as an enzyme that exhibits catalytic activity only in multimeric form. In a previous study, it has been shown that at temperatures above 50°C, multimers tend to dissociate, which leads to irreversible inactivation^[21]. Since immobilization using silica provided enzymes multipoint attachment and improved rigidity of the enzyme, thermal dissociation of silica-bounded GDH multimers could be prevented that lead to better activity retention at higher temperatures.

Free and silica immobilized GDH showed similar pH profiles which were, however, statistically significantly different over whole analyzed pH range (6–10), as illustrated in Fig. 3b. However, nanoSiO₂GDH and SBA15GDH exhibited about 10 and 15% enhancement of catalytic activity, respectively, particularly at basic conditions (8.5–10), compared to free catalysts. A shift of the pH optima from 8 (free GDH) to 8.5 (immobilized GDH) was also observed. Changes in the pH and temperature profiles could be explained by the fact that immobilization, in general, leads to conformational changes of the structure of the enzyme^[22]. These changes occur mainly as a result of ionization of side chains of active site amino acids. However, type, nature and functional group of the matrix also play a significant role. These factors lead to modifications of microenvironment around the active site of the enzyme and in consequence affect the pH and temperature profile of the immobilized biocatalysts^[23].

Evaluation of the thermal stability of the immobilized enzyme is a crucial step in determining practical applications of the produced biocatalytic systems. Binding of GDH to the silica support significantly improved thermal stability of the enzyme:

after 45 min of heating at 45°C, at pH 8, free enzyme maintained 42.5% of its activity, while nanoSiO₂GDH and SBA15GDH retained 47 and 53% of catalytic activity, respectively (Fig. 3c). Moreover, after 210 min of incubation under the same conditions, no catalytic activity was observed for free GDH whereas immobilized enzyme was more stable and retained about 30 and 35% of its activity when immobilized using nanoSiO₂ and SBA silica, respectively that might be explained by creation of a more suitable microenvironment after immobilization as reported also by Li et al.^[24]. Since drop in the relative activity of free GDH could be explained mainly by thermal and chemical inactivation of the biocatalyst, decrease in the activity of the immobilized enzyme could also partially reflect leakage of GDH from the matrix. Nevertheless, the higher relative activity noticed for SBA15GDH, as compared to nanoSiO₂GDH, is related to catalyst immobilization in the hexagonal pores of the support, which ensures better protection of the enzyme molecules against harsh reaction conditions.

Significant improvement in the stability of the immobilized GDH can also be observed in values of the inactivation constant (k_D) and enzyme half-life ($t_{1/2}$) (Fig. 3d). k_D and $t_{1/2}$ for free glucose dehydrogenase was found to be 0.0174 1/min and 39.8 min, respectively, whereas inactivation constants of nanoSiO₂GDH and SBA15GDH were, respectively, 4.5 fold and 7.2 fold (0.0038 1/min and 0.0024 1/min) lower than of those of the free enzyme. As a result, enzyme half-life was significantly improved and reached 182.4 min for nanoSiO₂GDH and 288.8 min for SBA15GDH. These findings are in agreement with data reported earlier by Twala et al. who immobilized GDH on functionalized ReSynTM polymer microspheres. However, in their study, immobilized enzyme half-life increased two fold after immobilization after incubation at 45°C compared to the free catalysts, while in the current study, enzyme half-life was improved much more (for about 4.5 fold). That is probably related to the more suitable chemical microenvironment created by the silica nanoparticles compared to polymeric support^[1].

Figure 3. (a) temperature profiles, (b) pH profiles and (c, d) thermal stability of free and silica immobilized glucose dehydrogenase (GDH). Thermal stability of free and silica immobilized GDH was examined under optimal temperature (45°C) and pH (8) conditions. Inactivation constants (k_D) were evaluated based on the linear regression slope. All data are presented as means $\pm$ standard deviation.

Effect of temperature and pH on activity and stability of free and immobilized GDH

The optimum temperature of free XDH was 40°C, whereas for nanoSiO₂XDH and SBA15XDH, the optimum shifted slightly upwards (45°C) (Fig. 4). Temperature profiles of free and immobilized XDH were also statistically significantly different. At temperatures below maximum, free enzyme showed higher activity compared with immobilized enzymes. However, when

temperature exceed 40°C, activity of free enzyme dropped sharply while immobilized XDH retained significantly higher activities. At 65°C nanoSiO₂XDH and SBA15XDH retained 69 and 64%, respectively, of their residual activity while free biocatalyst showed less than 50% residual activity. The higher temperature optimum recorded for immobilized XDH and the significant improvement of immobilized enzyme activity at higher temperatures are probably a result of creation of enzyme-matrix

interactions that form external enzyme backbones and increase rigidity of the biocatalyst^[25]. Such changes would have protected the active sites of the XDH against conformational changes and denaturation at higher temperatures, and thus help to maintain high catalytic activity.

All tested XDH systems exhibited their optimum at pH 8 (Fig. 3b). Moreover, their pH profiles were significantly different, however, particularly at pH values close to neutral (6.5–8.5), they were similar. Immobilization of XDH at basic pH (9–10) resulted in nanoSiO₂XDH and SBA15XDH showing about 10 and 20% higher activity, respectively, compared to the free catalyst. Under strongly acidic or basic conditions, ionic groups presented in the enzyme structure might be protonated or deprotonated, respectively. These changes result in formation of electrostatic repulsion between these groups, and hence destruction and degeneration of the enzyme active site and decrease in catalytic properties of the enzyme^[28]. Creation of the enzyme-matrix interactions which limit dissociation of enzyme subunits and stiffening the structure of the biomolecule after immobilization, caused that susceptibility of the enzyme to conformational changes in pH decreases, and leads to improvements of enzyme activity under harsh pH conditions.

Additionally, thermal stability of free and both silica-immobilized XDH was studied. Although, after immobilization activity

decreased (Table 2), stability of immobilized XDH was improved compared to the free enzyme (Fig. 4c). After 180 min of incubation at pH 8 and 40°C, free enzyme completely lost its activity while nanoSiO₂XDH and SBA15XDH retained more than 50% of their initial activity. Moreover, after 240 min of incubation at 45°C, XDH immobilized on silica nanoparticles or in hexagonal silica retained over 25 and over 30% of activity, respectively. As stability and catalytic activity of the enzyme after immobilization were improved, inactivation constant (k_D) and enzyme half-life ($t_{1/2}$) were also enhanced (Fig. 4d). k_D and $t_{1/2}$ of free enzyme reached 0.0237 1/min and 29.2 min, respectively, while after immobilization on silica nanoparticles the values were 0.005 1/min and 138.6 min, respectively. When hexagonal SBA silica was used as support, an even higher enzyme half-life (277.2 min) and lower inactivation constant (0.0025 1/min) were obtained. It might be explained by the fact that due to immobilization in the pores of the matrix, enzyme molecules are better protected compared to attachment of biocatalysts onto the surface of the matrix. Free enzyme on the other hand was subject to total inactivation due mainly to thermal and chemical and denaturation caused by heating and contact with base. However, negative effect of harsh reactive conditions on GDH and XDH was strongly hampered after enzyme immobilization due mainly to stabilization of the entire biocatalyst structure.

Figure 4. (a) temperature profiles, (b) pH profiles and (c, d) thermal stability of free and silica immobilized xylose dehydrogenase (XDH). Thermal stability of free and silica immobilized XDH was examined at optimal temperature (40°C for free enzyme and 45°C for immobilized enzymes) and pH (8) conditions. Inactivation constants (k_D) were evaluated based on the linear regression slope. All data are presented as means $\pm$ standard deviation.

Table 3. Kinetic parameters for free and silica immobilized GDH and XDH.

Analyzed parameter	freeGDH	nanoSiO ₂ GDH	SBA15GDH	freeXDH	nanoSiO ₂ XDH	SBA15XDH
K_M (mM)	16.1	21.8	28.7	0.31	0.34	0.45
V_{max}	7.7 U/mg	5.1 U/mg	4.1 U/mg	0.81 U/mL	0.70 U/mg	0.64 U/mg
k_{cat} (1/s)	79	77	75	27	21	19
k_{cat}/K_M (1/s*mM)	4.9	3.5	2.6	87.1	61.7	42.2

Kinetic parameters of free and immobilized enzymes

The K_M Michaelis–Menten constant and the V_{max} for free GDH were found to be 16.1 mM and 7.7 U/mg, respectively. After immobilization K_M increased and reached 21.8 and 28.7 mM for nanoSiO₂GDH and SBA15GDH, respectively. Simultaneously, V_{max} dropped 35 and 45% for nanoSiO₂GDH and SBA15GDH, respectively, compared to free catalyst. Nevertheless, it should be underlined that turnover number of immobilized biocatalysts was maintained almost unaltered as k_{cat} of nanoSiO₂GDH and SBA15GDH were 97 and 94% of that of free GDH, respectively (Table 3). Since turnover numbers of free and immobilized glucose dehydrogenase are comparable, increase in K_M value could be explained by the fact that after immobilization diffusional limitations occurred. As a result, active sites of the silica-bounded GDH are less accessible to the substrate and cofactor molecules, however, additional effects of cofactor-matrix and substrate-matrix interactions cannot be excluded either^[20]. Thus, as suggested earlier by Zhou et al., a higher concentration of substrates is required to enhance their interactions with immobilized catalysts^[27]. Moreover, as a result of enzyme attachment to the support, some of the active sites could be blocked, which reduces reaction rate and leads to drop in the maximum reaction velocity (V_{max}) [30]. Baron et al., who immobilized GDH from *Bacillus* onto DEAE-Sephadex modified by glutaraldehyde, also made similar observations. However, in their study K_M was 4-fold greater after immobilization and V_{max} was simultaneously almost 4-fold lower compared to the free enzyme^[20].

The K_M and V_{max} calculated for free XDH were 0.31 mM and 0.81 U/mL, respectively, and were comparable to those reported in our previous study where V_{max} has been found to be 0.56 U/mL [31]. The K_M of nanoSiO₂XDH and SBA15XDH were about 10 and 50% higher, respectively, compared with K_M of free XDH. However, the increase of K_M after immobilization observed for XDH was less significant than for GDH immobilized in this study. Simultaneously, a less significant drop of V_{max} was also noticed for immobilized XDH. These results could indicate that diffusional limitations that occurred after XDH immobilization were minimalized compared to GDH immobilization. This effect is probably related to the smaller molecular weight of XDH^[17]. Nevertheless, the turnover number of nanoSiO₂XDH and SBA15XDH were about 80 and 70%, respectively, of that of the free enzyme, which suggested that some conformational changes in the structure of active site amino acids occurred upon immobilization^[30]. These results support the data related to the retention of catalytic properties by immobilized XDH

presented above (Table 2). It also should be emphasized that, irrespectively of the used enzyme, higher values of K_M were noticed for enzymes immobilized using SBA silica, compared to nanoSiO₂, indicating lower substrate affinity to bounded GDH and XDH. It is related to the fact that using mesoporous silica (SBA 15) higher diffusional limitations occurred because biomolecules are immobilized mainly into its pores, as in case of nanoSiO₂ enzyme is attached onto the surface of the support, as presented in Fig. 1.

Conversion of biomass liquors

Investigation of stability of free and immobilized enzymes was carried out based on model solutions of xylose and glucose. Thus, to evaluate practical applications of the biocatalytic systems produced, for conversion of glucose xylose two different real biomass solutions, (i) a stream of monosaccharides (glucose, xylose, arabinose) obtained after nanofiltration of PTL and (ii) pretreated liquor (PTL), were used.

Figure 5. Conversion of (a) glucose to gluconic acid and (b) xylose to xylonic acid catalyzed by free and silica immobilized GDH and XDH, respectively, from monosaccharides solution and pretreated liquor (PTL). Dosage of enzyme: 0.5 mg of free or immobilized GDH or 200 U of free or immobilized XDH. All data are presented as means $\pm$ standard deviation.

Conversion of glucose to gluconic acid in the stream of monosaccharides by free GDH, nanoSiO₂GDH and SBA15GDH reached 76, 64 and 56%, respectively (Fig. 5a), corresponding to biocatalytic productivity values of 17.08, 14.41 and 12.56 mg of gluconic acid per 1 mg of free, nanoSiO₂ or SBA15 immobilized GDH, respectively (Table 4). By contrast, transformation of xylose from the same feed solution was lower and achieved 67, 57 and 52% for free, nanoSiO₂XDH and SBA15XDH, respectively (Fig. 5b), corresponding to a biocatalytic productivity of 0.180, 0.155 and 0.141 mg of xylonic acid per 1 U of free, nanoSiO₂ or SBA 15 immobilized XDH, respectively as shown in Table 4. Higher conversion of glucose to gluconic acid was mainly due to two factors. First of all, a greater amount of GDH was immobilized on silica carriers, moreover, attached GDH exhibited higher specific activity and activity retention compared to immobilized XDH. Second, concentration of glucose was about five times lower than xylose. The drop in conversion of both monosaccharides which was observed for immobilized enzymes compared to the free ones is related to decrease in enzyme activity upon immobilization, and has also been observed in other studies^[31,32].

Conversion of glucose and xylose presented in crude PTL (containing inhibitors) followed the same trend (Fig. 5a,b). However, compared to the stream of monosaccharides, in case of the PTL feed solution, about 15 and 20% lower values of conversion were achieved for glucose and xylose, respectively. This is directly related to the presence of inhibitors, such as formic and acetic acids or furans, in the feed solution, which could inhibit enzyme catalytic properties. The presence of trace amounts of phenols and monovalent (K⁺ or Na⁺) or divalent (Mg²⁺ or Ca²⁺) ions could also affect activity of both enzymes as reported earlier^[33]. Nevertheless, the results presented clearly show that investigated biocatalytic systems demonstrated efficient conversion of monosaccharides and therefore could find practical applications in the food industry as well as in biorefinery.

Reusability of the free and immobilized GDH and XDH

One of the great advantages of immobilization is enhancement of enzyme reusability in sequential conversion processes, which leads to decrease in process costs. As can be seen, free GDH and XDH gradually lost their catalytic properties: GDH was inactivated after four catalytic cycles using a monosaccharides stream and after four cycles using PTL as feed solution, while XDH was inactivated after three catalytic cycles irrespective of the feed solution used (Fig. 6). On the other hand, immobilized GDH retained around 70% of its initial activity and immobilized XDH maintained around 60% of its catalytic properties after three reaction cycles irrespective of the support material and feed solution. Moreover, after five reaction cycles, GDH and XDH immobilized onto SBA 15 surface retained over 50% of their initial activity during conversion of glucose and xylose from monosaccharides solution, and over 40% of initial catalytic properties when PTL was used as feed solution. This retention

of activity was higher than retention obtained when silica nanoparticles were used as carrier, as after five reaction cycles nanoSiO₂GDH and nanoSiO₂XDH maintained, respectively, only 43 and 39% of their catalytic properties with monosaccharides solution, and 38 and 33%, respectively, of their catalytic properties with PTL as feed solution.

The above results are in agreement with the data of biocatalytic productivity. After five consecutive reaction cycles, productivity of gluconic acid with the monosaccharides solution as the feed by free GDH reached 32.11 mg of gluconic acid per mg of enzyme. Productivity of nanoSiO₂GDH was about 1.6 fold higher (52.44 mg/mg), while productivity of SBA15GDH was about 1.4 fold higher (45.61 mg/mg) compared to the free enzyme (Table 4). Even higher increase in biocatalytic productivity of xylose was noticed using silica immobilized XDH and monosaccharides solution. After five reaction steps free XDH exhibited a productivity of 0.307 mg of xylonic acid per 1 unit of XDH, while productivity of nanoSiO₂XDH and SBA15XDH was 0.502 mg/U (1.7 fold) and 0.461 mg/U (1.6 fold), respectively. It should also be mentioned that lower values of biocatalytic productivity of gluconic and xylonic acid were observed using PTL as a feed solution; this result is directly related to higher concentration of glucose and xylose in PTL compared to in the stream of monosaccharides. These results confirm that reusability of the immobilized GDH and XDH was significantly improved. Nevertheless, it should be emphasised that irrespective of the feed solution used, during initial reaction cycles (up to third cycle), enzymes immobilized onto silica nanoparticles exhibited higher catalytic properties, but in further conversion steps, SBA 15 based systems showed higher activity. This phenomenon could be explained by the fact that enzyme immobilized in pores of hexagonal silica is better protected before inactivation and elution from the matrix, and thus its activity is less affected during the sequential conversion process. Higher retention of catalytic activity by enzymes used for conversion of both glucose and xylose from monosaccharides solution is probably related to the fact that the amount of enzyme inhibitors such as metal ions, inorganic acids and furans was significantly reduced compared to their presence in the PTL solution. Nevertheless, gradual decrease in enzyme activity after just a few catalytic cycles could be explained by several factors, such as enzyme inhibition by product, structural modification of the enzymes as well as by catalyst inactivation caused by their thermal and pH denaturation^[34]. It should be also mentioned that due mainly to adsorption interactions, some enzyme leakage from the matrix (up to 30% after last cycle) also occurred as a contributory factor. Earlier studies reported that glucose-6-phosphate dehydrogenase encapsulated in silica-based hydrogels retained less than 40% of its initial activity just after one reaction cycle^[35], as in another study, 40% of activity retention was observed after seven reaction cycles when glucose-6-phosphate dehydrogenase was immobilized in alginate^[23].

Table 4. Biocatalytic productivity of free and immobilized GDH (mg of gluconic acid per mg of enzyme) and free and immobilized XDH (mg of xylonic acid per U of enzyme) after five consecutive catalytic cycles.

Analyzed parameter	Biocatalytic productivity (GDH in mg/mg) and (XDH in mg/U)					
	freeGDH	nanoSiO ₂ GDH	SBA15GDH	freeXDH	nanoSiO ₂ XDH	SBA15XDH
Monosaccharides	32.11	52.44	45.61	0.307	0.502	0.461
PTL	31.12	51.10	39.08	0.204	0.336	0.279

Figure 6. Reusability of the free and silica immobilized GDH and XDH evaluated based on consecutive conversion of glucose and xylose, respectively, using monosaccharides solution after nanofiltration (a) and (b) and PTL (c) and (d) as feed solution. All data are presented as means $\pm$ standard deviation.

Conclusions

Our study provides proof-of-concept for the immobilization of glucose dehydrogenase (GDH) and xylose dehydrogenase (XDH) on silica nanoparticles and hexagonal mesoporous SBA15 silica, and application of the obtained biocatalytic systems for effective conversion of glucose and xylose from biomass liquors. Specific activity of free GDH and XDH (assessed as NADH consumption during reduction of glucose and xylose, respectively) was comparable and reached 42.2 U/mg and 46.8 U/mL, respectively. After immobilization, both enzymes exhibited high catalytic activity retention, but better loadings and activity retention were obtained for GDH and XDH immobilized on the surface of silica nanoparticles than on the mesoporous silica particles, which confirms that type of support material affects properties of the biocatalytic systems obtained.

Nevertheless, on both types of silica supports, the immobilized enzymes attained improved thermal stability both silica-immobilized enzymes are characterized by improved thermal stability, with half-life of SBA15GDH and SBA15XDH being increased 7.25 and 9.5 fold, respectively as compared to free biocatalysts, after incubation for four hours at 45°C. However, immobilization did not affect the pH optima of any of the two enzymes, regardless of the type of silica immobilization material, and the immobilized enzymes showed the same high activity over a broad range of pH (over 50% activity at pH range 8–10). Robustness at higher pH, above the pK_a of gluconic and xylonic acid, is of particular interest, because this feature facilitates simple separation of the gluconic and xylonic acid from reaction mixture even by a simple membrane filtration. Additionally, the immobilized enzymes exhibited improved reusability; not only was their catalytic activity retained after five consecutive reaction cycles but the biocatalytic productivity was improved about 1.6 fold by nanoSiO₂GDH and nanoSiO₂XDH compared to the free

enzymes. The data thus clearly shown that immobilization of GDH and XDH is an attractive approach for efficient transformation of glucose and xylose into valuable products. Hopefully, the data may stimulate further development of the use of monosaccharide dehydrogenases in biorefining and separation of monosaccharides in genuine biomass liquors.

Experimental Section

Chemicals and reagents

Commercially available silica nanopowder of 10–20 nm particle size (nanoSiO₂) and mesoporous SBA 15 silica (<150 μm particle size) with hexagonal pore morphology and pore size up to 20 nm (SBA15) used in this study were provided by Sigma-Aldrich (Steinheim, Germany). Tris-HCl buffer, phosphate buffer, β-nicotinamide adenine dinucleotide hydrate (NAD⁺), β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH), D-glucose, D-xylose and glucose dehydrogenase from *Pseudomonas* sp. (GDH) (EC 1.1.1.118) were purchased from Sigma-Aldrich (Steinheim, Germany). Xylose dehydrogenase (XDH) (EC 1.1.1.175) was provided by Megazyme (Bray, Wicklow, Ireland). All chemicals were of analytical grade and were used as received from the suppliers without further purification.

Real liquors

The pretreated liquor (PTL) used in this study was obtained after combined acid and hydrothermal pretreatment of the biomass. For pretreatment, diluted sulfuric acid and high temperature steam (180°C) were injected in the reactor containing soaked wood for a 10 min. After pretreatment, the liquid fraction was separated from the solids by pressing in reactor. Prior to use, the PTL was filtered using the microfiltration membrane GR40PP (MWCO 100 kDa) at 4 bar in an Amicon 8050 (Millipore, Burlington, MA, USA) stirred cell to clarify the solution. The concentration of monosaccharides, glucose, xylose and arabinose was 13.3 g/L, 55.2 g/L, and 2.1 g/L, respectively. The stream of monosaccharides without inhibitors (inorganic acids and furans) containing glucose (10.3 g/L), xylose (48.8 g/L) and arabinose (1.9 g/L) was obtained after nanofiltration of PTL at 40 bar using a NF90 nanofiltration membrane (MWCO 200–400 Da) in a stainless steel stirred cell (HP4750, Sterlitech Corporation, Kent, WA, USA).

Immobilization of GDH and XDH

For the immobilization of GDH and XDH, 50 mg of silica nanoparticles or silica SBA 15 was immersed in 2 mL of enzyme solution in phosphate buffer at pH 7 containing 0.15 mg (30U) of GDH or 30U of XDH. The immobilization was carried out by incubation of the silica support (nanoSiO₂ or SBA 15) with each enzyme solution for 3 h at 4°C in an IKA KS 4000i control incubator (IKA Werke GmbH, Germany) with mixing at 200 rpm. The immobilized enzymes (nanoSiO₂GDH, SBA15GDH, nanoSiO₂XDH, SBA15XDH) were recovered from the solution by centrifugation at 4000 rpm for 15 min (Sigma 4K15, Sigma Laborzentrifugen GmbH). The protein content in the samples after immobilization (amount of immobilized enzyme (mg/g)) was determined by the Bradford method (eq. 1) as the difference in the initial enzyme dosage protein and the concentration of protein present in the supernatant after immobilization^[36] and considering mass of the support material, according to eq. 1. From the results of the Bradford method, immobilization yield (%) was calculated (eq. 2).

$$\text{Amount of immobilized enzyme} = \frac{c_i V_i - c_s V_s}{m_{\text{support}}} \quad (\text{eq. 1})$$

$$\text{Immobilization yield (\%)} = \frac{c_i V_i - c_s V_s}{c_i V_i} \cdot 100\% \quad (\text{eq. 2})$$

where c_i and c_s denote concentration of enzyme before and after immobilization (mg/mL or U/mL), V_i and V_s denote volume of the solution

(mL) before and after immobilization, and m_{support} is the mass of the support material (g).

GDH and XDH activity

Activity assays for GDH and XDH were performed spectrophotometrically during kinetic reduction of NAD⁺ to NADH, as presented below, using a standard calibration curve of NADH. One unit of free or immobilized enzyme activity was defined as the quantity of enzyme required to produce 1 μM of NADH per minute under the assay conditions. Specific activity values (U/mg) of free and immobilized enzymes are presented as initial enzyme activity retained per unit mass of enzyme, and per unit mass of enzyme and solid support, respectively. The activity retention (%) of immobilized enzyme was defined as the percentage activity of the immobilized GDH and XDH compared to the catalytic activity of free enzymes. All measurements were done in triplicate and error bars are presented as means ± standard deviation.

GDH activity assay

Catalytic activity of free and immobilized GDH was evaluated spectrophotometrically by measuring the absorbance at λ=340 nm (Shimadzu UV1280, Shimadzu, Japan) during kinetic reduction of NAD⁺ to NADH. The reaction was carried out for 2 min in a styrene cuvette with a volume of 1 mL of reaction mixture containing 20U of free or immobilized GDH, 3 mM NAD⁺ and 50 mM D-glucose in Tris-HCl buffer at pH 8, 45°C.

XDH activity assay

Activity of free and immobilized XDH was determined spectrophotometrically by following changes in the absorbance at λ=340 nm during kinetic reduction of NAD⁺ to NADH^[37]. The reaction was carried out in a styrene cuvette with a volume of 1 mL of reaction mixture containing 30 U of free or immobilized XDH, 3 mM NAD⁺ and 70 mM D-xylose in Tris-HCl buffer at pH 8 for 2 min at 45°C.

pH profiles of free and immobilized enzymes

The pH profiles of free and immobilized GDH and XDH were examined using the methodology described above, at pH values ranging from 6 to 10 at 45°C. The pH of the solution was adjusted using 0.1 M HCl and 0.1 M NaOH. All measurements were made in triplicate and error bars are presented as means ± standard deviation.

Temperature profiles of free and immobilized enzymes

The temperature profiles of free and immobilized glucose dehydrogenase and xylose dehydrogenase were evaluated based on the above-mentioned methodology at temperatures varying from 30°C to 60°C in steps of 5°C, at pH 8. Prior to spectrophotometric measurements, free and immobilized enzymes were incubated at the desired temperature for 30 min. All measurements were made in triplicate and error bars are presented as means ± standard deviation.

Stability of free and immobilized enzymes

Stability of both free and immobilized enzyme over time was evaluated after incubating the native and immobilized enzymes for 240 min under optimum pH and temperature conditions. For free and silica immobilized GDH, the optima were 45°C and pH 8, while for free and silica immobilized XDH, the optima were pH 8 and 40°C and pH 8 and 45°C, respectively. The relative activity of free and immobilized enzyme was further determined after a specified period of time. The initial activity of free GDH and XDH was defined as 100% activity. The inactivation constant (k_D), and half-life ($t_{1/2}$) were evaluated based on the linear regression slope. All measurements were made in triplicate and error bars are presented as means ± standard deviation.

Kinetic parameters of free and immobilized enzymes

Examination of the kinetic parameters - the Michaelis-Menten constant (K_M), maximum reaction rate (V_{max}), turnover number (k_{cat}) and specificity constant (k_{cat}/K_M) - of free and immobilized GDH and XDH was

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performed based on the model enzymatic reactions (see section GDH and XDH activity), using various concentrations of NAD⁺. The kinetic parameters were calculated based on Hanes-Wolf plots under optimum assay conditions.

Glucose and xylose conversion

In the presented study, two biomass liquors were used which contained monosaccharides only (monosaccharides stream) and monosaccharides with inhibitors (PTL) (see section Real liquors). The reaction was performed in glass tubes. For the reaction, 1 mL of monosaccharides solution or PTL was used to which was added 0.5 mg of free or immobilized GDH or 200 U of free or immobilized XDH and 10 mM of NAD⁺. Prior to the reaction, the pH of reaction mixture was adjusted to 8 and to 8.5 for GDH and XDH, respectively using 0.1 M NaOH. After 30 min of the process, the reaction was terminated by addition of 2 mL of 1 M HCl and the mixture was subjected to HPLC analysis. Conversion of glucose to gluconic acid by GDH and xylose to xylonic acid by XDH was evaluated based on the results of High Performance Liquid Chromatography (HPLC) and calculated using eq. 3. Shimadzu Corp. (Japan) equipment was used in the HPLC-analysis (LC-20AD, DGU-20A3, SIL-20AC, SCL-10A, CTO-10A). The column system consisted of an Aminex HPX-87H Ion Exclusion Column (300 mm × 8.7 mm) (Bio-Rad) and a security guard (H+) pre-column. The temperature was 63°C, the eluent was 4 mM H₂SO₄ and the flow rate was 0.6 mL/min. Carbohydrates and acids were detected using a refractive index detector (RID-10A). Samples were diluted with the eluent to obtain concentrations of monosaccharides, and acids in the range of 0.05–5 g/L and 0.025–3 g/L, respectively.

$$\text{Conversion (\%)} = \frac{C_F - C_P}{C_F} \cdot 100\% \quad (\text{eq. 3})$$

where C_F and C_P denotes xylose or glucose concentration (g/L) in feed solution and after enzymatic conversion, respectively.

Reusability study of free and immobilized enzymes

The reusability of the free and immobilized GDH and XDH was examined by measuring conversion of xylose and glucose in the stream of monosaccharides and pretreated liquor, according to methodology presented in the above section, over five consecutive reaction cycles. After each conversion cycle, the immobilized GDH and XDH was separated from the reaction mixture by centrifugation and washed with Tris-HCl buffer solution before the next cycle. Free enzyme was separated from the reaction mixture by nanofiltration at 4 bar in an Amicon 8050 (Millipore, USA) using an NF90 membrane. The conversion of glucose and xylose by GDH and XDH, respectively, in the first catalytic cycle was defined as 100%. The biocatalytic productivity of free and immobilized enzyme was expressed as mass of product formed (mg) by mass of the enzyme used.

Statistical analysis

Statistically significant differences were determined by one-way ANOVA performed in SigmaPlot 12 (Systat Software Inc., USA) using Tukey's test. Statistical significance was established at a $p < 0.05$ level.

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Immobilization of glucose and xylose dehydrogenase using silica-based support materials improved enzymes stability and reusability as well as enhanced efficient conversion of biomass components in bioreactors, under mild conditions.

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monosaccharides by immobilized
glucose dehydrogenase and xylose
dehydrogenase