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Multienzymatic conversion of monosaccharides from birch biomass after pretreatment

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ABSTRACT

This study presents proof-of-concept validation of a multi enzymatic biocatalytic system, capable of producing monosaccharides from a birch biomass pretreated liquor with simultaneous cofactor regeneration. To improve the stability of the proposed biocatalyst, co-immobilization of NAD^+-dependent xylose dehydrogenases, glucose dehydrogenase, and NADH-dependent 3-hydroxybutyrate dehydrogenase was performed on mesoporous silica SBA-15. The results of the physicochemical analysis confirmed the effectiveness of enzymes deposition on the support material and changes in the surface area and porous structure of silica before and after the process. Process conditions in which the three-enzymes system allowed to achieve the highest efficiency of the conducted reactions were optimized at 20 °C, pH 7, 90 min of process duration, 400 mM of levulinic acid addition and ratio of NAD^+:NADH 1:5. Results from this study demonstrate excellent xylonic acid, gluconic acid and 4-hydroxyvaleric acid production at conversion rate of 98.7%, 95.6% and 99.2%, respectively. In addition, the high stability and reusability of the developed biocatalytic system was confirmed based on tests in ten successive catalytic cycles and the storage time of 10 days at 4 °C. At the end of the tests, co-immobilized enzymes were capable of catalyzing the process with >70% efficiency. The presented data confirmed the effectiveness of co-immobilization, improvement of enzyme properties including their high reusability, as well as indicated the application potential of the proposed multienzymatic system in the conversion of components of pretreated biomass solutions.

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1. Introduction

Enzymes have been widely used as biocatalysts in various technological processes due to excellent catalytic and physicochemical properties (Jankowska et al., 2021; Noreen et al., 2021; Bachosz et al., 2022a). Nevertheless, the variety of their optimal environmental conditions and high costs have significantly contributed to their limited use of enzymes in their native forms for commercial applications (Iqbal et al., 2022; Zdarta et al., 2022). Therefore, it is necessary to immobilize enzymes on a carefully selected support to address the above-mentioned problems (Nguyen et al., 2020; Zdarta et al., 2021). The course of a correctly carried out enzyme deposition depends on the appropriate selection of the method of its immobilization, as well as on the type of support material (Mohamad et al., 2015). It should be highlighted that

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the development of innovative support materials with new and/or improved properties play a key role due to necessity of their suitable coordination with enzymes, which is related to surface chemistry, pendent functional entities and the ease in tunability of material. A consequence of this approach might be creation of robust biocatalytic structures, which significantly expand the practicality of using enzymes (Bilal and Iqbal, 2019; Liu et al., 2021). Moreover the characteristics of the support material have a significant impact on the cost of the reaction and on the properties of the immobilized enzyme. Regardless of the method of protein deposition, there is a very wide range of materials that can be used as supports, so first of all, it should be noticed that the price and features that will improve the efficiency of the biocatalytic system are the most important issue (Guisan et al., 2022). The support should be distinguished by high mechanical resistance, which is essential to maintain the stability, due to the protection of the enzyme structure against harsh process conditions, which in turn allows to keep the high activity of the protein (Zdarta et al., 2018). Another important feature is the ability to stably bind the enzyme to the support. The material should have a large number of functional groups, i.e. hydroxyl and amino groups, showing high reactivity towards biomolecules, which prevents the enzyme from leaching (Santos et al., 2015). The selection of the support is also influenced by its insolubility in the reaction medium (Nguyen and Kim, 2017). Important parameters of the support material are also the pore size of the material and its surface area. Smaller particles provide a larger surface area for protein deposition, in relation to the volume of their pores, which translates into an increase in the effectiveness of immobilization (Kothalawala et al., 2022). The size of the pores is also important because the presence of pores smaller than or equal to the enzyme molecules can lead to the immobilization of the biocatalyst on the outer surface of the material, resulting in faster washout of the protein and its lower protection (Matte et al., 2016). In addition, the size and number of pores define the size of the surface area, which is desirable due to the fact that it results in a limited diffusion resistance (Matte et al., 2016). Most of the above-mentioned requirements can be satisfied by silica materials that have a large amount of hydroxyl groups on the surface and, consequently, can be used in adsorption and covalent immobilization. Additionally, the silica is characterized by a well-developed surface and porous structure, which results in high sorption capacity. Importantly, these properties are tunable due to the fact that it is possible to select a type of silica that will allow the enzyme to be deposited on the surface of the material and/or in its pores. Moreover, the characteristic features of this inorganic support are thermal, chemical and mechanical resistance (Zucca and Sanjust, 2014). This seems to be the decisive factor while the selection of support material for the immobilization of enzymes, which will be applied in the conversion of real solutions containing various chemical compounds.

Biomass liquors from the pretreatment of lignocellulosic biomass are a mixture of various compounds that are formed after depolymerization or conversion of the main components of the raw materials (Vu et al., 2020). Examples of these compounds include cellulose, hemicellulose and lignin (Kumari and Singh, 2018). Glucose, xylose, arabinose, mannose, vanillin, phenols, furan derivatives or organic acids are just some of the potential products of biomass pretreatment to compounds with lower molecular weight and high utility (Madadi et al., 2017; Lee et al., 2022). These products have important practical application in many different fields of science, everyday life and industry (Deng and Amarasekara, 2021). Among these pretreatment products, sugars have been the most widely used in the food processing, pharmaceutical, chemical, and biotechnology industries (Cho et al., 2020; Kartik et al., 2021). Most of compounds from pretreatment can undergo further transformations, especially glucose and xylose, which constitute the largest part of the solutions after biomass pretreatment. The bioconversion processes of sugars obtained from biomass are largely based on oxidation and reduction reactions involving enzymes from the group of oxidoreductases (Manavalan et al., 2021). For the proper functioning of oxidoreductases, the presence of cofactors — low molecular weight non-protein compounds, which ensure maximum catalytic properties of proteins and prevent undesirable modifications in their structure, is required (Trisolini et al., 2019).

There has been a growing interest in enzymatic reactions for developing novel and more efficient biocatalytic systems (Sai Prethi et al., 2022). In these biocatalytic systems, cofactors also play a significant role. Nevertheless, in biocatalytic synthesis, cofactors must be used with suitable stoichiometric ratio, which limits their application in large-scale processes due to the high costs associated with the supply of subsequent portions of the cofactor, which is consumed during the reaction (Wang et al., 2021; Wu et al., 2021). Therefore, it is extremely important to develop methods of cofactor regeneration, which will not only reduce the costs of the processes, but also enable the use of cofactor depended-enzymes on a large industrial scale (Wichmann and Vasic-Racki, 2005).

Biomass from weed such as aspen (*Populus sp.*), birch (*Betula sp.*) and willow (*Salix sp.*) is an important resource in Europe (Hagner et al., 2020). In this study, the bioconversion of monosaccharides (xylose and glucose) from real birch pretreated liquor to xylonic acid and gluconic acid was performed using NAD^+-dependent xylose dehydrogenase (XDH) and glucose dehydrogenase (GDH). Furthermore, in this system simultaneous regeneration of the enzymatic cofactor was possible by introducing co-substrate, which was levulinic acid and its conversion to 4-hydroxyvaleric acid using NADH-dependent 3-hydroxybutyrate dehydrogenase (3HBDH). Additionally, in order to improve the stability and resistance of the proposed multienzymatic system, three biocatalysts were co-immobilized using silica SBA-15. During the research, tests were carried out on various forms of enzymatic systems, as well as the optimal process conditions for the effective operation of the developed biocatalyst were determined in order to define the most suitable process conditions and biocatalytic system produced. Finally, the designed XDH/GDH/3HBDH system has been tested in successive catalytic cycles and its storage stability has been defined. It should be clearly highlighted that all tests were carried out in order to demonstrate the potential of the proposed biocatalytic system in the simultaneous bioconversion of the components of pretreated liquor and the regeneration of the cofactor. Moreover, to the best of our knowledge, the proposed multienzymatic system is the first concept of the use of three synergistic and co-immobilized enzymes in the conversion of the solution after birch biomass pretreatment.

Table 1
Final composition of the pretreated birch liquor.

Components	Concentration in solution (g/L)
Xylose	48.80
Acetic acid	12.05
Glucose	10.35
Furfural	2.70
Arabinose	1.94
Formic acid	0.92
5-Hydroxymethylfurfural	0.30
Inorganic salts	0.1
Phenols	<0.1

2. Materials and methods

2.1. Chemicals and reagents

D-xylose, D-glucose, levulinic acid, xylonic acid lithium salt, gluconic acid, 4-hydroxyvaleric acid, β-nicotinamide adenine dinucleotide hydrate (NAD^+), β-nicotinamide adenine dinucleotide reduced disodium salt hydrated (NADH), *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 50 mM TAPSO buffer at pH 7 as well as SBA-15 silica were supplied by Sigma-Aldrich (USA). Glucose dehydrogenase from *Pseudomonas* sp. (GDH, EC 1.1.1.47; 200 U/mg) and 3-hydroxybutyrate dehydrogenase (3HBDH, EC 1.1.1.30; 3 U/mg) were supplied by Sigma-Aldrich (USA), and xylose dehydrogenase in the form of solution (XDH, EC 1.1.1.175; 60 U/mL) was purchased from Megazyme (Ireland).

2.2. Composition of the real liquor

The pretreated liquor was obtained after birch biomass pre-treatment by a combination of acidic and hydrothermal methods. Birch biomass was soaked in a dilute solution of sulfuric acid and high-temperature steam (180 °C) for 10 min. Then, the liquid phase was separated from the solid phase by pressing the wooden residue in the reactor. To clarify the solution, filtration was performed using a microfiltration membrane GR40PP (MWCO: 100 kDa) at 4 bar in Amicon 8050 reactor (Millipore, Burlington, MA, USA). The final stage in the preparation of the real liquor was to minimize the content of inorganic acids, furans and phenols, which may be potential inhibitors of further processes. Next, nanofiltration was performed using a NF90 membrane (MWCO: 200-400 Da) at 40 bar in a stainless steel cell (HP4750, Sterlitech Corporation, Kent, WA, USA). The final composition of the real solution was shown in Table 1. Due to the highly acidic pH of the real liquor, the pH was raised to 7 with 2 M NaOH prior to carrying out the enzyme processes.

2.3. Co-immobilization of enzymes

Enzymes containing 20 U of xylose dehydrogenase, 10 U of glucose dehydrogenase and 30 U of 3-hydroxybutyrate dehydrogenase were added to 2 mL (or 100 mg) of SBA-15 silica in TAPSO buffer at pH 7. For mixed biocatalytic systems, one enzyme is in free from (solution) while the remaining enzymes were co-immobilized the same activities as above-mentioned were used. Co-immobilization was carried out in a thermomixer for 2 h with a constant stirring speed of 300 rpm at 21 °C. After co-immobilization, the mixture was centrifuged and the supernatant was removed. The remaining silica was transferred to a watch glass and allowed to dry at ambient temperature for 24 h.

2.4. Physicochemical and structural analysis of the obtained biocatalyst

To determine the effectiveness of the enzyme co-immobilization and to characterize the biocatalytic systems a multiple analytical techniques were used. FTIR analysis was performed using a Bruker Vertex 70 IR (Germany) spectrometer in ATR (Attenuated Total Reflectance) mode over a wavenumber range of 4000–400 cm^{-1} (resolution 0.5 cm^{-1}). Energy-dispersive X-ray microanalysis (EDS) was carried out using the EDS analyzer contained in the Tescan (Czech Republic) scanning electron microscope with Gamma-Tec tooling from Priceton Inc. Moreover ASAP 2020 physisorption analyzer (Micromeritics Instrument Co., Norcross, GA, USA) was used in order to define the parameters of the porous structure of silica materials. The surface area was determined according to Brunauer–Emmett–Teller (BET) algorithm, meanwhile mean pore size and total pore volume were calculated based on the Barrett–Joyner–Halenda (BJH) method.

2.5. Comparison of effectiveness of bioconversion with different enzymatic systems

Five different biocatalytic systems made of: (i) free enzymes, (ii) free GDH and co-immobilized XDH/3HBDH, (iii) free XDH and co-immobilized GDH/3HBDH, (iv) free 3HBDH and co-immobilized GDH/XDH and (v) three co-immobilized proteins were used in the bioconversion of monosaccharides from pretreated liquor, for comparison. In each individual enzymatic system, co-immobilization was performed according to the above-mentioned methodology. In order to carry out simultaneous bioconversion of biomass components and cofactor regeneration, levulinic acid with a concentration of 46.40 g/L as well as cofactors in the ratio of NAD^+:NADH 1:5 were added to the reaction mixture consists of various enzyme forms and pretreated liquor. Bioconversion of monosaccharides was performed for each tested system under the same process conditions (300 rpm, ambient temperature, process duration 90 min). After the end of the reaction, the samples were centrifuged, and then the supernatant was analyzed by GC–MS. The obtained results were used to determinate biocatalytic productivity (mM/U), expressed as mole of product formed (mM) by 1 unit (U) of the selected enzyme used, as well as productivity of acids (%), which was calculated on the basis of initial and final substrate concentration.

2.6. Bioconversion of real solution components catalyzed by co-immobilized enzymes

The reaction mixture consisted of pretreated liquor (mainly xylose and glucose), levulinic acid, NAD^+, NADH and co-immobilized together xylose dehydrogenase, glucose dehydrogenase and 3-hydroxybutyrate dehydrogenase. All processes were carried out in a thermomixer with temperature control, mixing of 300 rpm for 90 min. First, the effect of pH and temperature on the efficiency of acids production was checked. The pH of the reaction mixture was tested in the range of 5–9 (at 20 °C), while the temperature at the values: 10, 15, 20, 25 and 30 °C (at pH 7). Then, the effect of the concentration of added levulinic acid on the efficiencies of the bioconversion processes were determined. The following concentrations of levulinic acid were used: 50, 100, 200, 400, 500 mM. The effect of various ratios of cofactors NAD^+:NADH (1:1, 1:2, 1:4 and 1:5) was also determined. The time curves for the production of xylonic acid, gluconic acid and 4-hydroxyvaleric acid were examined under optimal process conditions (20 °C, pH 7, 400 mM levulinic acid, ratio of NAD^+:NADH 1:5) for 90 min. On the basis of these results, the rate of formation of xylonic acid, gluconic acid and 4-hydroxyvaleric acid over time was calculated from the linear parts of the curves. Additionally, the reusability and storage stability of the developed enzymatic system was checked in 10 successive catalytic cycles and over storage at 4 °C for 10 days. All processes were carried out at ambient temperature, pH 7, with the addition of 400 mM levulinic acid and the ratio of NAD^+:NADH 1:5 for 90 min. After each completed process, the collected samples were centrifuged, and the obtained supernatant was analyzed using GC–MS to determine the concentration of substrates and reaction products and to determine stability of the biosystems.

2.7. Chromatographic analysis of products

All collected samples were properly prepared and analyzed by means of GC–MS measurements to determine the concentration of xylonic acid, gluconic acid and 4-hydroxyvaleric acid in each sample. The obtained post-reaction mixtures were dried with a SpeedVac (Eppendorf, Germany) concentrator at 37 °C. Then the samples were derivatized by adding 50 μL BSTFA and incubating for 3 h at 70 °C. The sample prepared in this way was dissolved in 100 μL of hexane and transferred to chromatographic vials. The chromatographic analysis was carried out on a GCxGC-TOFMS chromatograph (Pegasus 4D, Leco Corp., USA) according to the procedure and conditions described in our previous studies (Bachosz et al., 2022b). The analytes were identified on the basis of the obtained mass spectra using ChromaTof software (v4.51.6.0, Leco Corp., USA) and the NIST library. Quantification of xylonic acid, gluconic acid and 4-hydroxyvaleric acid was carried out using a previously prepared calibration curve, based on the correlation between the peak area in the chromatogram and the standard compound concentration.

2.8. Statistical analysis

All measurements were made in triplicate. Error bars are presented as means ± standard deviation. Statistically significant differences were determined using Tukey's test by one-way ANOVA performed in SigmaPlot 12 (Systat Software Inc., USA). Statistical significance was established at the level $p<0.05$.

3. Results and discussion

3.1. Characterization of biocatalytic system

To determine the functional groups present on the surface of the tested materials and, consequently, indirectly verify the effectiveness of the enzyme co-immobilization process, the FTIR analysis was performed (Fig. 1). In the spectrum for the pure support there are visible signals characteristic for the bonds occurring in the silica structure. At the wavenumber of approx. 1100 cm^{-1} there is a signal generated due to the stretching vibrations of Si–O–Si bonds, while at approximately

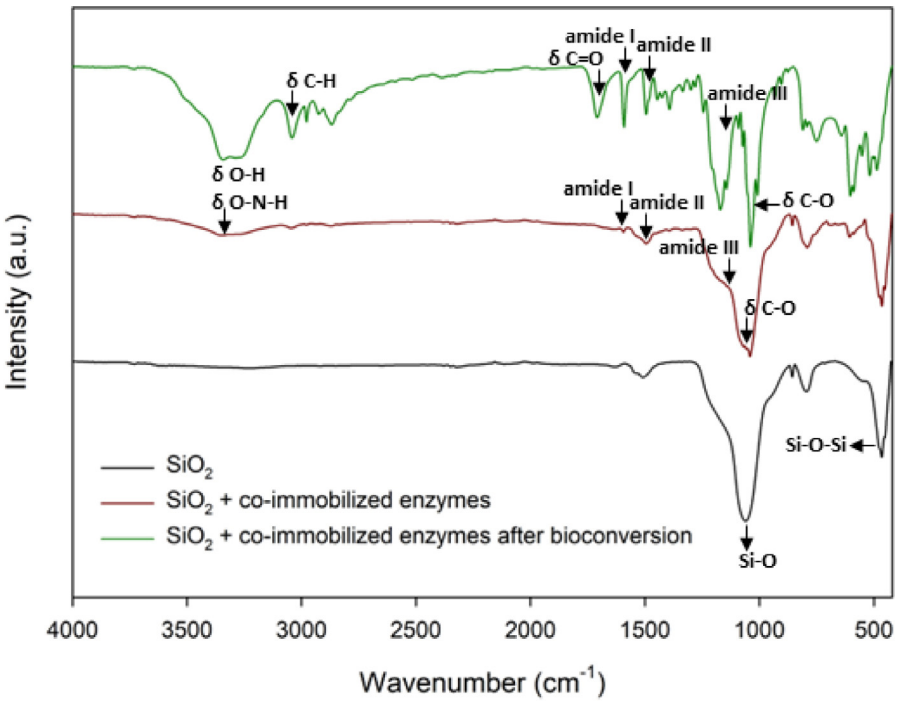

Fig. 1. FTIR spectra of the silica before and after co-immobilizations as well as after bioconversion.

800 cm^{-1}, a peak appears from the vibrations of stretching Si–OH bonds. Finally, a specific signal, characteristic for silica, can be seen at a wavenumber of approx. 470 cm^{-1} and comes from the stretching vibrations of the Si–O bonds. On the other hand, from the FTIR spectrum of the support with co-immobilized enzymes on its surface, it can be concluded that the co-immobilization of three proteins was correctly performed, which confirms the presence of signals assigned to bonds contained in the structure of biocatalysts (Qu et al., 2014). First of all, at the wavenumber with the maximum at 3400 cm^{-1}, a new peak appeared, which is characteristic for the stretching vibrations of O–H and N–H bonds in the enzyme structure. In addition, there is also a noticeable signal at 1650 cm^{-1}, which is generated by the stretching vibrations of the amide I bonds. It should be noted however that the lower intensity of signals characteristic for enzymes is due to the significant predominant of silica in the content of the sample (Katiyar et al., 2021). Additionally, after completing of the monosaccharide bioconversion, FTIR analysis of the biocatalyst was also performed. As can be seen, in the spectrum of this sample, there are many new signals, related to the C=O bonds, aromatic and aliphatic –CH groups and the aromatic ring including C–C and C=C bonds, that make it impossible to clearly verify the presence of enzyme in the material. Nevertheless, this result points to the fact that other components of the real solution or the products of reactions can adsorb onto the silica surface and might affect enzymatic performance.

As it is commonly known, silica is characterized by a well-developed surface area and porous structure, as well as a high sorption capacity, therefore it is a good choice to be used as a support in the immobilization of enzymes. Additionally, silica SBA-15 allows on the deposition of enzyme on its surface and in the pores, thus stability of the proteins is relatively high even in the harsh process conditions (Jin et al., 2018; Lin et al., 2021).

Using the BET and BJH algorithms, it was calculated that pure silica has a surface area of 24.28 m^2/g, whereas its pore size is 16.25 nm. The obtained data correspond to the values presented in the safety data sheet provided by the supplier. After carrying out the same measurements for the material after the enzymes co-immobilization process, there was a significant decrease in the value of the surface area of silica, which was found to be 5.74 m^2/g. In addition, the pore volume changed as well as the size of the pores. A decrease in the value of these three parameters indicates the deposition of enzymes on the silica surface as well as in its pores, and confirms effective co-immobilization of proteins (Garcia et al., 2022) (see Table 2).

Finally, energy dispersion X-ray analysis (EDS) allowed to indicate which chemical elements compose the surface of the analyzed samples. Fig. 2 presents the EDS spectra together with the tables with the percentage share of individual elements on the surface of all tested materials. The analysis of the results obtained for pure SBA-15 silica shows that its surface consists mainly of two elements: oxygen (55.3%) and silicon (43.3%), as well as trace amounts of nitrogen, sulfur and zinc, which might be an impurity remaining after the synthesis of the material or generated during the preparation of the sample for analysis. Nevertheless, after the co-immobilization of enzymes, the elemental composition of the material changed, due to the addition of nitrogen and sulfur. The contents of nitrogen and sulfur increased from 1.4%

Table 2
Porous properties of silica before and after enzymes co-immobilization.

Sample	Surface area (m^2/g)	Total pore volume (cm^3/g)	Mean size of pores (nm)
SiO_2	24.28	0.098	16.25
SiO_2 + co-immobilized enzymes	5.74	0.022	10.25

Table 3
Productivity and biocatalytic productivity of various organic acids depending on different biocatalytic systems.

Biocatalytic system	Productivity (%)			Biocatalytic productivity (mM/U)		
	Xylonic Acid	Gluconic acid	4-Hydroxy valeric acid	Xylonic acid	Gluconic acid	4-Hydroxy valeric acid
Free enzymes	91.3	91.3	84.2	15.1	5.48	11.2
Free GDH + co-immobilized XDH/3HBDH	94.6	88.3	96.3	15.6	5.30	12.8
Free XDH + co-immobilized GDH/3HBDH	83.6	87.9	88.7	13.8	5.28	11.8
Free 3HBDH + co-immobilized XDH/GDH	91.0	95.2	84.1	15.0	5.71	11.2
Co-immobilized enzymes	98.7	95.6	99.2	16.3	5.74	13.2

to 2.0% and 0.1% to 2.0%, respectively, confirming protein deposition on the silica surface. Both nitrogen and sulfur are in multiple bonds, i.e. sulfide bridges or amide bonds, which are the basis of the structure of immobilized dehydrogenases (Zhang et al., 2020). Moreover, the results obtained for a sample of the biocatalyst material after the bioconversion of monosaccharides from a real solution are interesting. It can be noticed that there has been a significant increase in the content of sulfur, nitrogen as well as zinc and consequently a decrease in the amount of silicon and oxygen. These results correspond to the data from FTIR analysis and may also suggest adsorption of various chemicals, other than the enzymes, presented in pretreated liquor on the silica surface.

3.2. Characterization and comparison of different enzymatic systems

To define the form of the biocatalyst that would be most effective in monosaccharides conversion with the simultaneous action of three enzymes, tests were carried out on five different enzymatic systems, in native, co-immobilized or mixed forms. When all three dehydrogenases were co-immobilized to silica substrate, the highest productivities of xylonic acid, gluconic acid and 4-hydroxyvaleric acid of over 98%, 95% and 99%, respectively were observed in Table 3. The highest efficiency of the mentioned biocatalyst was also confirmed by the calculated biocatalytic productivity of each enzyme, which was much higher (especially for XDH and 3HBDH) than the values obtained with other systems. This results might be related to the improvement of the biocatalyst stability, and, consequently, the reduction of the influence of the process environment on the catalytic activity of enzymes.

Acid production by free enzymes are also high, taking into account the presence of many inhibitors in the pretreated liquor, in particular acetic acid, which is a smaller molecule than other compounds present in the mixture and can compete with xylose, glucose or levulinic acid for the availability of the active site of enzymes (Sun et al., 2019). Nevertheless, acid production by free enzymes is the lowest among all tested biocatalytic systems. These results clearly show a large effect of the reaction medium on the activity of native biocatalysts and suggest better protection of the co-immobilized by the silica support. Interestingly, relatively high productivity values were obtained for the mixed-form enzymatic systems, but it should be noted that in each of the three cases, the lowest process efficiencies were obtained for the reaction in which the biocatalyst was in free form. Thus, it is another proof of the necessity to apply the co-immobilization process, which can significantly improve the stability and resistance of the biocatalytic system, as well as provide protection for enzymes against rapid loss of activity (Pietricola et al., 2021). Therefore, further research focused on determining the optimal process conditions were performed for a system with three co-immobilized enzymes using silica.

3.3. Catalytic properties of the co-immobilized XDH/3HBDH/GDH system

Time curves are necessary to determine changes in the amount of substrates and products during the reaction. Therefore it was decided to define the optimal reaction time that will allow to obtain the highest productivity of xylonic acid, gluconic acid and 4-hydroxyvaleric acid. As can be seen from the curves shown in Fig. 3a, there was a rapid and significant increase in the amount of products in the reaction mixture during the first 30 min of the process, as the productivity of all three acids was above 68% at this point. During the next 60 min of the process, the efficiency of the

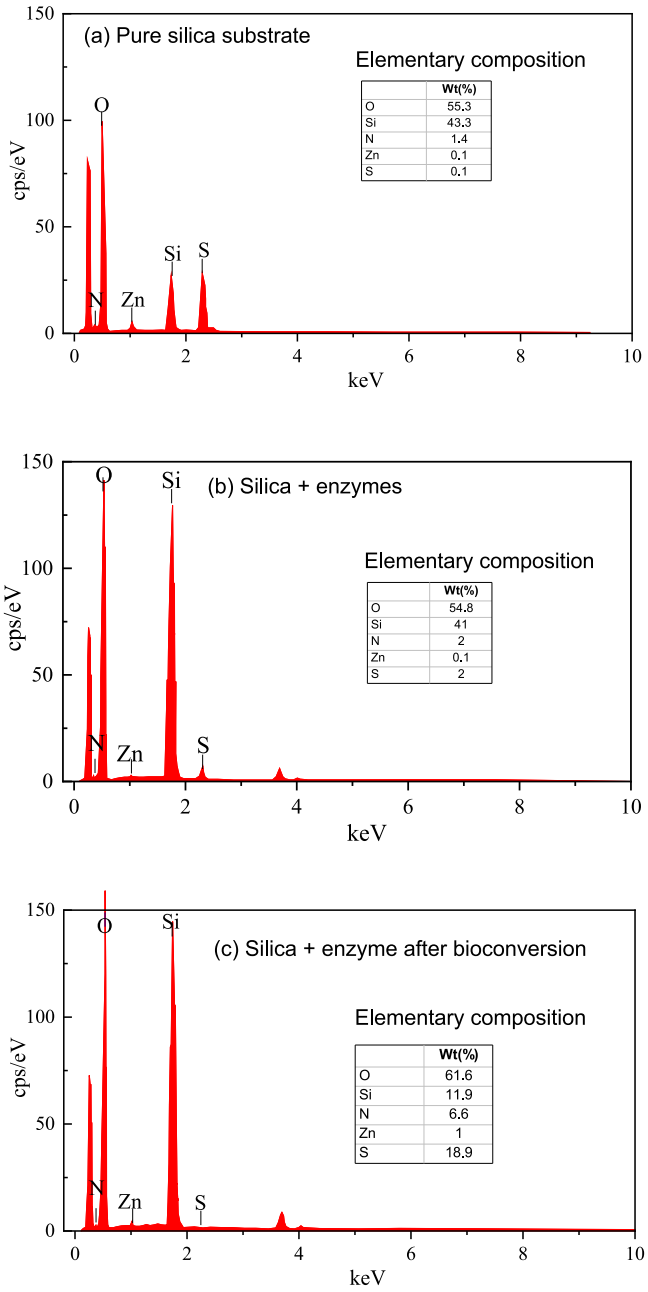

Fig. 2. EDS spectra of: (a) pure silica, (b) material with co-immobilized enzymes and (c) biocatalytic system after bioconversion.

bioconversion increased steadily until the highest yields were obtained after 90 min. Further prolongation of the reaction duration did not increase acids productivity. Hence, it seems that the optimal time for monosaccharide bioconversion is 90 min. Additionally, attention should be paid to the rates of all three conducted reactions, which have to form a synergistic system in order to create the enzymatic cofactor regeneration system (Zhang et al., 2011). As is known, the bioconversion of levulinic acid to 4-hydroxyvaleric acid is responsible for the production of the certain amount of NAD^+, which is then used by XDH and GDH to produce xylonic acid and gluconic acid. The rate of 3HBDH catalyzed reaction was found to be 10.62 mM/min, while the value of this parameter for the xylose and glucose conversion processes is 7.56 mM/min and 1.43 mM/min, respectively. Thus, it seems that the production of NAD^+ with the above-mentioned rate is sufficient to ensure the constant supply of this form of cofactor in the reaction mixture, as well as the simultaneous combined actions of XDH and GDH allow for the production of the appropriate amount of NADH, which is necessary for

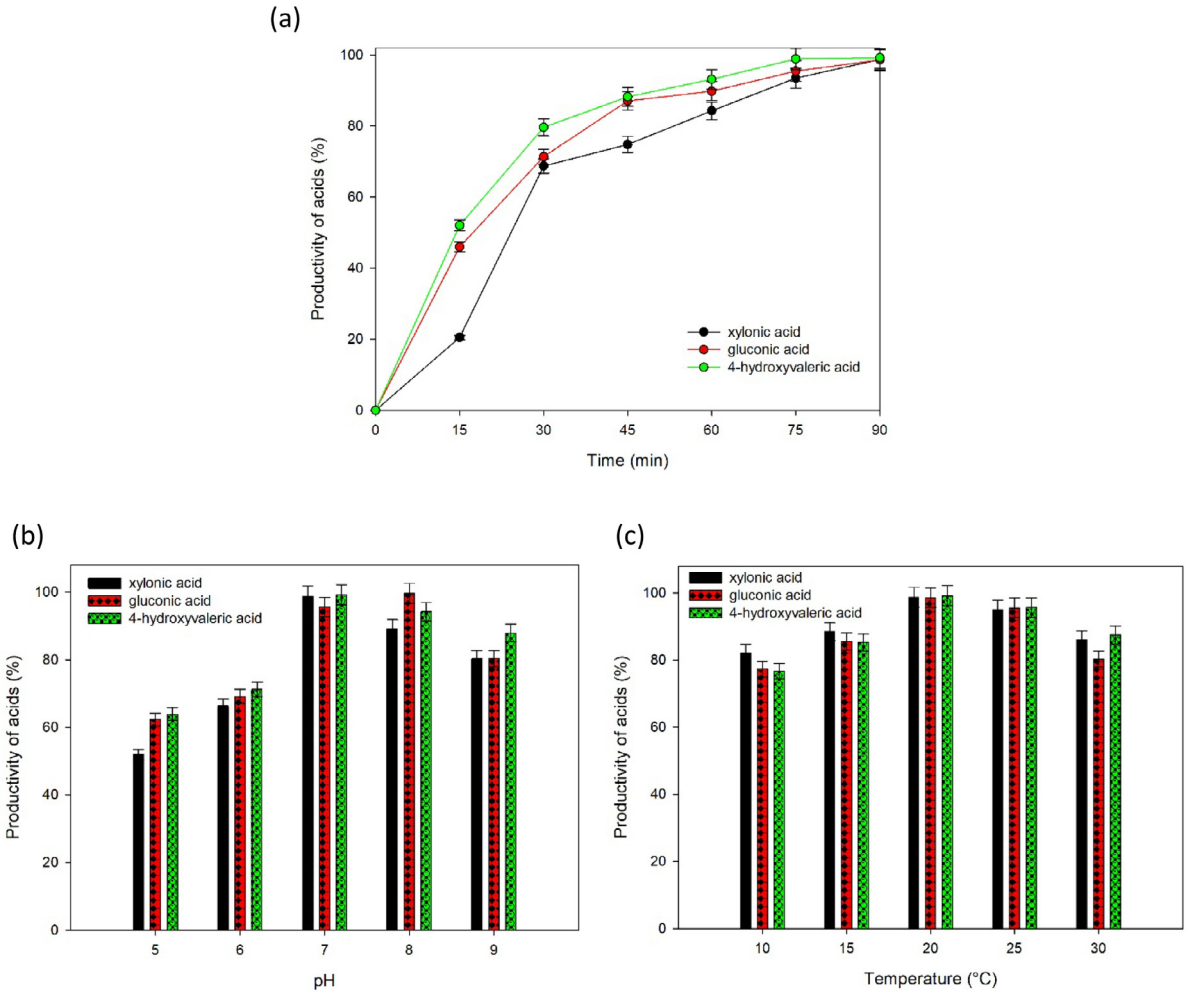

Fig. 3. Productivity of xylonic acid, gluconic acid and 4-hydroxyvaleric acid by co-immobilized XDH/3HBDH/GDH depending on the: (a) time as well as (b) pH and (c) temperature with the addition of 400 mM levulinic acid and the ratio of NAD^+:NADH 1:5 for 90 min. All data are presented as mean ± standard deviation of 3 experiments.

the proper functioning of 3HBDH. Therefore, it might be concluded that the regeneration of the enzymatic cofactor should be effective, which is additionally evidenced by the obtained high efficiencies of acids production.

Individually, XDH, GDH and 3HBDH are characterized by maintaining high catalytic activity at a temperatures in the range of 20–30 °C (Yeon et al., 2013; Stolarczyk et al., 2020). However, in the case of the pH of the reaction mixture, xylose dehydrogenase prefers a neutral environment, whereas glucose dehydrogenase shows the highest stability at pH 8 (Nygard et al., 2014; Gao et al., 2016). 3HBDH is more universal due to the fact that it can be used in slightly acidic and neutral or slightly alkaline environments (Uchino et al., 2007). Hence, it is important to carry out tests that would indicate the optimal process conditions for the synergistic work of these three enzymes. Fig. 3b shows the results obtained for tests to determine the optimal pH of the reaction mixture. As can be observed, the highest productivity of xylonic acid and 4-hydroxyvaleric acid was obtained for the system operating at pH 7, while the highest efficiency of gluconic acid production was at pH 8, which confirmed the individual preferences of biocatalysts.

Nevertheless, it should be noted that the highest cumulative productivity was found when a reaction was conducted at pH 7, whereas in the case of the biocatalytic system working at pH 8 the productivity of xylonic acid and 3-hydroxyvaleric acid dropped below 90%. This result might be explained by the higher susceptibility of XDH and 3HBDH to the influence of changing the reaction environment compared to GDH, especially due to the fact that their optimal conditions for catalytic activity are at neutral pH. Additionally, it should be emphasized that in an alkaline environment (pH 8 and pH 9) it is possible to carry out all bioconversion processes with relatively high efficiency. In contrast to an acidic environment (pH 5 and pH 6), in which it was impossible to carry out the production of all three acids with an efficiency higher than 70%. Therefore, it seems extremely important to initially neutralize the pH of the pretreated liquor with the use of NaOH, which was done before each tested process. Moreover, while examining the effect of temperature on the efficiency of

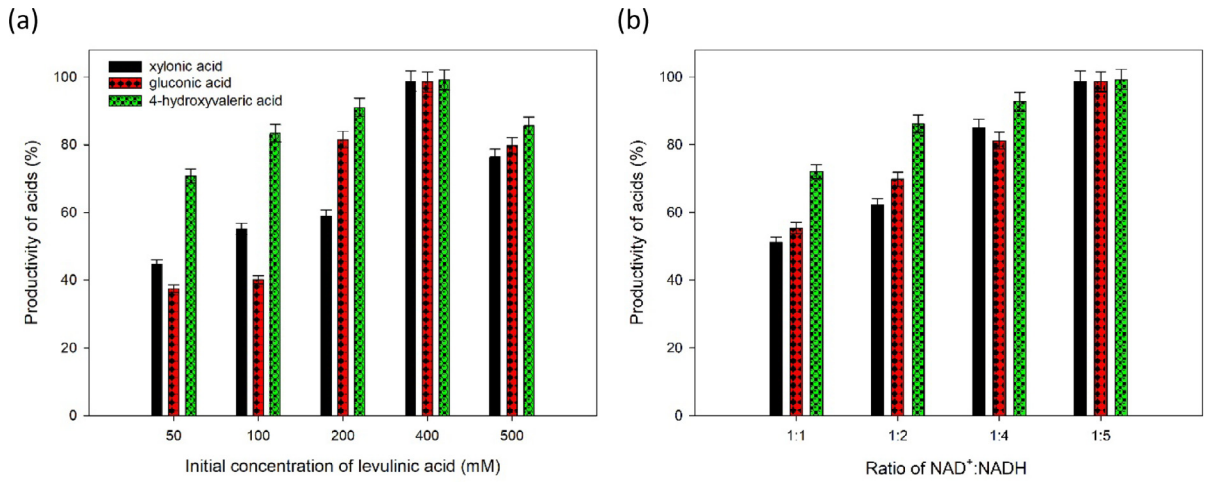

Fig. 4. Productivity of xylonic acid, gluconic acid and 4-hydroxyvaleric acid by co-immobilized XDH/3HBDH/GDH depending on the: (a) initial content of levulinic acid and (b) ratio of cofactors. All data are presented as mean ± standard deviation of 3 experiments.

the proposed biocatalytic system (Fig. 3c), it was noticed that relatively high productivity of acids was obtained in the entire temperature range studied, which indicates a high thermal resistance of the developed biocatalyst. The highest productivity for all three acids was obtained for the system operating at a mild temperature of 20 °C, while the lowest production (about 75%) took place at 10 °C probably due to insufficient enzyme activation. Based on the analysis of the obtained results, it was decided that the optimal conditions for the XDH/GDH/3HBDH system are 20 °C and pH 7, and therefore in the further part of the research all processes were carried out under these conditions.

Due to the fact that the initial concentration of xylose and glucose was determined by their content in the pretreated liquor, it was necessary to select the appropriate concentration of the levulinic acid addition, as well as the amount of enzymes and the ratio of cofactor forms. Based on previous studies on cofactor regeneration systems using two parallel reactions, it was proved that coupled XDH and 3HBDH or GDH and 3HBDH work with the highest efficiency in a ratio 1:1 (Zheng et al., 2011; Jia et al., 2018). Therefore, in the course of the present tests, it was decided that the enzymatic activity ratio would depend on the substrate concentrations and the protein ratio in previous research. Due to the fact that the concentration of xylose in the reaction system is much higher than the concentration of glucose, it was settled to introduce 20U XDH and 10U GDH for their conversion. However, the amount of 3HBDH was selected in the ratio of 1:1 to the both above-mentioned enzymes, therefore 30U of this enzyme was added and consequently the ratio of all enzymes was found to be: 2:1:3 for XDH, GDH and 3HBDH, respectively. After examination of optimal enzymes ratio, it was decided to check the effect of the initial levulinic acid content as well as the ratio of cofactors on the efficiency of the bioconversion processes. Fig. 4a shows the results of the research for the variable levulinic acid content. As can be seen, the system in which initially 400 mM of levulinic acid was found is characterized by the highest productivity of each organic acid. Relatively high bioconversion efficiencies were also achieved for the reaction mixture containing the addition of levulinic acid, which concentration was 500 mM. Additionally, it should be emphasized that in each of these systems with levulinic acid amount different than 400 mM, the production of 4-hydroxyvaleric acid was much more effective than the production of xylonic acid and gluconic acid, which might result from the insufficient amount of NAD^+ in the reaction mixture and, consequently, inhibition of the main monosaccharide bioconversion reactions by improperly functioning NAD^+-dependent enzymes (Veesar et al., 2015). This conclusion is clearly supported by the results of the effect of cofactor ratio tests, which were shown in Fig. 4b.

In the case of developing a multienzymatic system, it is necessary to determine the appropriate amount of cofactor forms in the reaction mixture. The results obtained clearly show that for monosaccharides bioconversion to be effective, at the beginning of the process a significant amount of NADH in the system is necessary, due to the fact that the highest productivity of all three acids was obtained for the NAD^+:NADH ratio of 1:5. It can be seen that the less of NADH in the reaction system was, the lower acids production yields were achieved. First of all, this might be due to the amount of enzymes as well as the amount of substrate, and additionally, the rate of the reaction (Su et al., 2021). In addition, the fact that it can be too small amount of NADH in the system can also play a significant role. Consequently levulinic acid bioconversion as well as cofactor regeneration do not run efficiently and at some point during the course of process NAD^+ could be not enough in the reaction mixture, while this form of cofactor is necessary for the proposed biocatalytic system to properly convert monosaccharides from biomass.

Interestingly, Jin et al. (2019) performed the reduction of xylose to xylitol with simultaneous bioconversion of glucose to gluconic acid using NADPH-dependent xylose reductase and $NADP^+$-dependent glucose dehydrogenase in a ratio of 1:3. As was shown in the results, the most effective process was carried out in the presence of NADPH: $NADP^+$ cofactors in the ratio of 1:4, which confirms the importance of selecting appropriate amounts of cofactor forms in relation to the amount of enzymes and substrates.

3.4. Stability of co-immobilized enzymes

For scaling up, it is essential to check the potential of the developed multi-enzymatic system in successive catalytic cycles, as well as to assess the storage stability. Therefore, the designed biocatalyst was tested in 10 reaction cycles that were carried out under optimal conditions. As can be seen in Fig. 5a, after the completion of ten consecutive cycles, the biocatalytic system could still efficiently perform the bioconversion process with a productivity of xylonic acid, gluconic acid and 4-hydroxyvaleric acid above 80%. Moreover, it should be emphasized that the storage of the biocatalyst for 10 days at 4 °C did not significantly affect stability, as it was possible to obtain acids production efficiencies above 70%. These results confirm the stability of the developed multi-enzymatic system for extended operation in a scaled up process. The enzymes are not only protected by the support, but also maintains catalytic activity. Similar conclusions were presented by Zhou et al. (2022), who co-immobilized His-tagged old yellow enzyme and glucose dehydrogenase on modified SiO_2 nanoflowers with simultaneous cofactor regeneration. The developed biocatalytic system was able to carry out seven consecutive reaction cycles while maintaining the productivity on the level of 50%. Moreover, the old yellow enzyme could catalyze the reduction of 3-methyl-2-cyclohexen-1-one with an efficiency of about 90% and the bioconversion of glucose was carried out with the yield of about 85% after storage of the biocatalyst for 10 days at 4 °C. Therefore, it seems that the developed XDH/GDH/3HBDH system has great potential in further research also due to the practical aspects of its application.

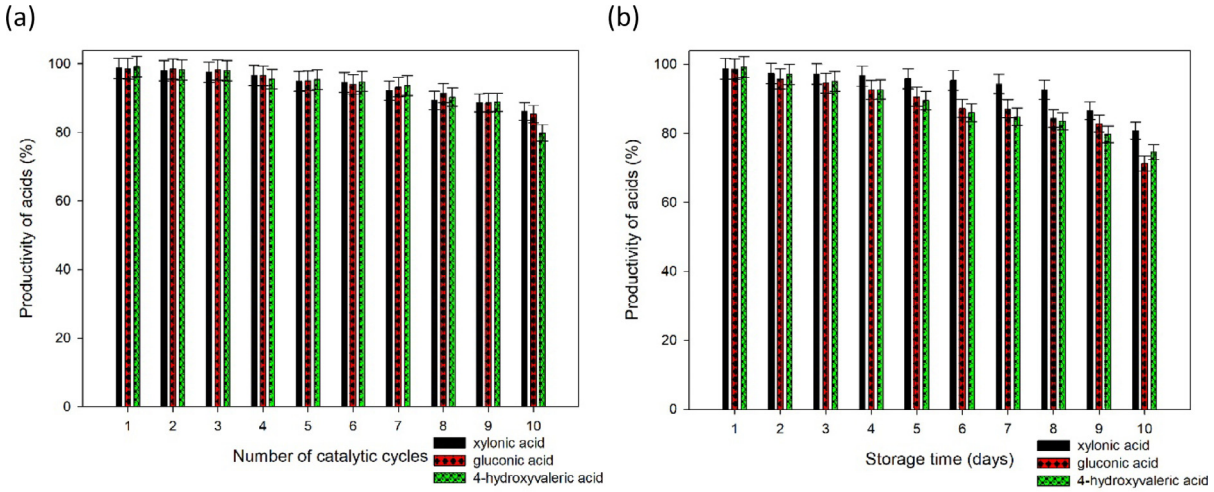

Fig. 5. (a) Reusability of the co-immobilized XDH/3HBDH/GDH and (b) storage stability of biocatalytic system. All data are presented as mean ± standard deviation of 3 experiments.

3.5. Mechanism of enzymes co-immobilization and bioconversion processes

In the tested multienzymatic system, the synergistic course of all the conducted reactions is extremely important, as well as the amount of reactants and products in the reaction mixture. It cannot be ignored that the interactions between enzymes and the support, which allow to maintain the stability of the developed biocatalyst, also have a great influence. Therefore, as can be seen in Fig. 6, an attempt was undertaken to present the basic mechanism of enzyme co-immobilization using silica SBA-15, as well as the assumptions underlying the process of the cofactor regeneration system.

Because co-immobilization was performed using adsorption approach, it was possible that physical interactions between the support and enzymes were generated, including van der Waals forces, adsorption interactions, and hydrogen bonds. Importantly, above-mentioned bonds are rather weak, so their presence do not change the structure of the enzymes, and as a consequence, the active site of the proteins is protected against disturbing and the activity of the enzymes is maintained (Jesionowski et al., 2014). Additionally, it should be mentioned that many hydroxyl groups are present on the surface of silica, which proves its high hydrophilicity and readiness to generate interactions (Zdarta et al., 2018). It should be emphasized that in our studies on the FTIR spectrum after co-immobilization, slight shifts of signals characteristic for amide bonds towards higher wavelengths, compared to the spectrum of native enzymes (data not shown) are noticeable. This result suggests that between the hydroxyl groups (derived from SiO_2) and amide bonds (part of the enzymatic amino acids residues), mainly hydrogen bonds were formed, which allowed for effective co-immobilization of XDH, GDH and 3HBDH on the support. Nevertheless, it is also possible to deposit the enzymes in the pores of the support, not only to bind it to the surface. Matching the pore diameter of the silica and the size of the enzymes can additionally contribute to the protection of the co-immobilized enzymes (Califano and Costantini, 2020). Silica SBA-15 is a mesoporous

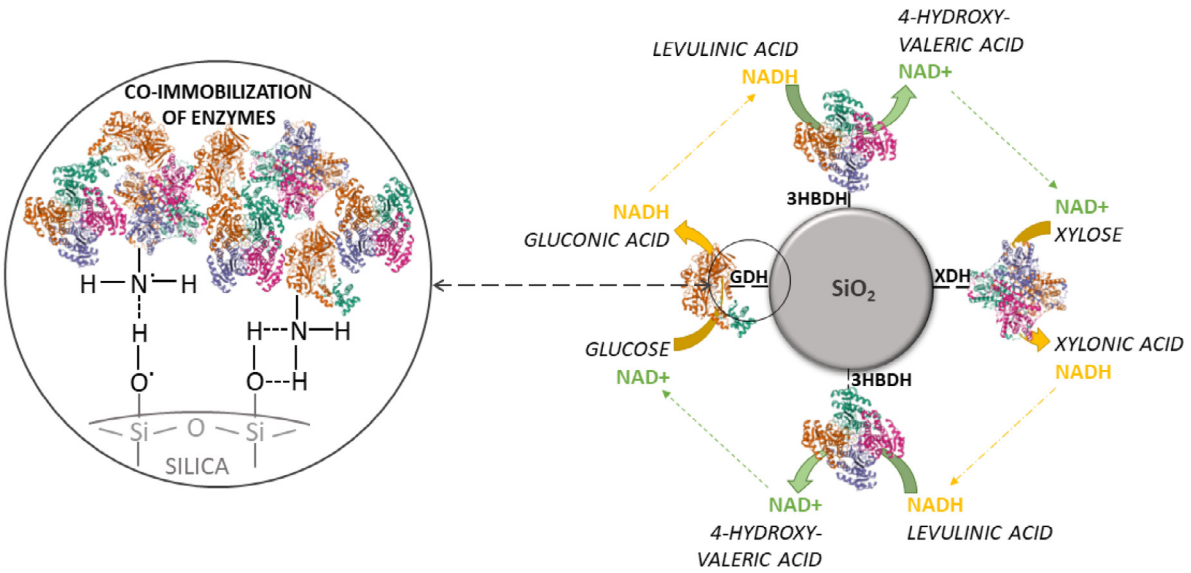

Fig. 6. Mechanism of enzyme co-immobilization and bioconversion processes with simultaneous cofactor regeneration.

material with a pore diameter equal to several nanometers. Therefore, it was found that its porous structure adequately fixed the size of the co-immobilized enzymes which were found to be about 7 nm, 6 nm and 3 nm for XDH, GDH and 3HBDH, respectively (Johnsen and Schonheit, 2004; Lu et al., 2014; Hyun et al., 2016). These assumptions correspond to the results obtained during the analysis of the porous structure of the material after co-immobilization using the BET method.

In the development of the presented biocatalytic system, an equally important issue as the enzyme co-immobilization mechanism was to ensure optimal operation environment of the three reactions carried out in parallel. It seemed to be necessary to create a compromise between bioconversion rates and cofactor regeneration. It was possible to identify the effectiveness of the multienzymatic system by maximizing the biocatalytic productivity of enzymes, balancing the rates of all reactions, as well as a high value of the total turnover number in relation to the cofactor (Marpani et al., 2017). As mentioned, levulinic acid was introduced into the solution after the pre-treatment of the birch, so that it was possible to regenerate the NAD^+ consumed in the bioconversion of xylose and glucose. In catalytic cycle of 3-hydroxybutyrate dehydrogenase, NADH was reduced to NAD^+, which was used by XDH and GDH and then oxidized to NADH. As a result of those processes, again 3HBDH could use the appropriate form of a cofactor and the regeneration process could continue. The cofactor regeneration cycle is shown in Fig. 6 using arrows (green arrow shows use of NAD^+ for monosaccharide bioconversion; yellow arrow shows application of NADH in cofactor regeneration via 4-hydroxyvaleric acid production).

The greatest influence on the effectiveness of the cofactor regeneration system has the synergistic action of the enzymes present in the reaction mixture, which should work at such a rate that none of the cofactor forms is missing. Therefore, it is important to balance the rate of the formation course of all three organic acids. The highest value for the reaction rate was calculated for the process involving 3HBDH, NAD^+ formation, and was found to be 10.62 mM/min. Nevertheless, the xylose conversion rate is slightly lower (7.56 mM/min), while still in the reaction system NADH is also produced during the process with GDH at a rate of 1.43 mM/min. Thus, it seems that the rates of the production of three organic acid are balanced and allow for effective cofactor regeneration. Additionally, it should be mentioned that the value of the total turnover number (TTN), calculated as the number of moles of formed product to the total amount of cofactor added to the reaction, was found to be 8814. This result indicates the high efficiency of the proposed multienzymatic system, especially when compared to other chemical or electrochemical methods of cofactor regeneration, which allow to achieve relatively low TTN values (<100) (Liu and Wang, 2007). Finally, it should be emphasized that the co-immobilized enzymes made it possible to carry out monosaccharide bioconversion with simultaneous cofactor regeneration with higher biocatalytic productivity than native proteins, which is the added value of the proposed multienzymatic system. Thus, on the basis of the obtained results, it can be confirmed that the designed biocatalytic system can be effective and meet the objectives assumed during the research.

4. Conclusions

In summary, xylose dehydrogenase, glucose dehydrogenase and 3-hydroxybutyrate dehydrogenase co-immobilized using silica SBA-15 could effectively convert monosaccharides from pretreated liquor to their corresponding acidic forms. Physicochemical properties of enzyme-immobilized on silica before and after the protein deposition process were

systematically characterized. Then the optimal conditions for bioconversion of sugars were determined to be 20 °C, pH 7, 90 min of process duration, 400 mM of initial levulinic acid concentration and ratio of NAD^+:NADH 1:5. Under these conditions xylonic acid, gluconic acid, and 4-hydroxyvaleric acid were produced with high efficiencies of 98.7%, 95.6% and 99.2%, respectively. Moreover, it has been proved that the system with three co-immobilized enzymes achieves higher yields of organic acid production than systems with mixed forms of proteins or native biocatalysts. Additionally, the high stability and resistance of the developed biocatalytic system was confirmed, based on the tests of successive catalytic cycles and storage stability. After ten catalytic cycles, it was still possible to produce organic acids with an efficiency of >80%, while after 10 days of storage the productivity was over 70%. In summary, results from this study demonstrate the potential of our proposed XDH/GDH/3HBDH biocatalytic system for utilizing birch biomass to produce valuable bio-products. Further research is expected for system scaling up and techno-economic assessment.

CRediT authorship contribution statement

Karolina Bachosz: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft. **Jakub Zdarta:** Conceptualization, Writing – review & editing, Supervision. **Long D. Nghiem:** Writing – review & editing. **Teofil Jesionowski:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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