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A Novel Cysteine-Functionalized M_xO_y Material as Support for Laccase Immobilization and a Potential Application in Decolorization of Alizarin Red S

Agnieszka Kołodziejczak-Radzimska * and Teofil Jesionowski

Faculty of Chemical Technology, Institute of Technology and Chemical Engineering, Poznan University of Technology, Berdychowo 4, PL-60965 Poznan, Poland; teofil.jesionowski@put.poznan.pl

* Correspondence: agnieszka.kolodziejczak-radzimska@put.poznan.pl

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Abstract: Immobilization process improves the enzyme properties, like stability, activity, selectivity or specificity. In the study, a novel cysteine-functionalized M_xO_y (ZrO_2, SiO_2) material was used as a support for the immobilization of laccase from *Trametes versicolor*. The proposed matrix was prepared using a simple sol-gel method. The cysteine was introduced during the synthesis of a sample. Additionally, the obtained supports were modified with glutaraldehyde. The basic properties of the prepared cysteine functionalized ZrO_2 and SiO_2 were determined using spectroscopic, thermal, porous, electrostatic and elemental analysis. Furthermore, the obtained biocatalytic systems were used as catalysts in the oxidation of sulfonic acid. Catalytic and kinetic parameters were determined based on the proposed model reaction. Next, laccase immobilized on ZrO_2- and SiO_2-based materials were, for the first time, utilized in the decolorization of Alizarin Red S. In that process, the influence of duration, pH and temperature on the efficiency of decolorization was evaluated. The results show that the proposed biocatalytic systems offer good specific activity (ca. 19 U/mg) and activity retention (ca. 77%). Importantly, they can be successfully used in the decolorization of Alizarin Red S with high efficiency (above 95%).

Keywords: L-cysteine; SiO_2; ZrO_2; laccase; catalysis; kinetic; decolorization

1. Introduction

The enzyme immobilization is a powerful tool in biocatalyst design, improving protein properties. A proper immobilization can increase stability and activity of enzyme under conditions far from the physiological ones, enzyme selectivity and specificity (using substrates far from the physiological ones), enzyme purity and sensitivity to inhibition, as well as resistance to chemicals. The support properties, the active group presence in the support and enzyme should be considered in immobilization protocol [1].

Cysteine ((R)-2-amino-3-mercaptopropionic acid, *Cys*) is a branched amino acid which contains three functional groups: thiol, amino and carboxyl. The thiol groups can be used to create supports with disulfide bonds. The thiol groups can form stable disulfide bonds; they may bind with metals by coordinate bonding or remain in reduced form [2,3]. Besides, thiol groups play an important role in the synthesis and functionality of metal nanoparticles, because of their high affinity to the particle surface [4]. Additionally, cysteine prevents the aggregation of nanoparticles and enables the attachment of enzymes on the particles' surface. The amino and carboxyl groups present in *Cys* are suitable for the immobilization of enzymes. Furthermore, because of the presence of thiol groups, cysteine is used in the pharmaceutical industry (for drug delivery) and the food industry (as a food additive) [5,6]. Cysteine as a natural antioxidant in several biological processes, including protein

synthesis, metabolism, and detoxification. Due to its thiol groups, cysteine can easily be oxidized to cystine, a dimeric amino acid. This reaction is reversible and allows the control of a wide range of biological activities and protein structures, and, therefore, the determination of cysteine in biological matrices and pharmaceutical preparation is highly important [5]. The cysteine is using in food chemistry as a reducing agent in production of French bread, crackers and cookies. The cysteine is effective in preventing browning of fruit juice during concentration. They also prevent the development of off-flavor in stored orange juice. In addition, in flavor chemistry, cysteine is an important source of sulfur in a variety of aromas [6].

Metal oxides (M_xO_y: SiO_2, ZrO_2, ZnO, Fe_2O_3, Al_2O_3, etc.) are commonly used as supports for the immobilization of enzymes because of their good thermal and chemical stability, in addition to excellent mechanical resistance [7–10]. These materials are easy to synthesize, which makes them relatively cheap. Moreover, the surface of M_xO_y particles can be modified by various groups, enabling the attachment of enzymes to the surface. The thiol groups present in cysteine have a strong tendency to be adsorbed onto the surfaces of certain metals [11]. According to reports in the literature, cysteine has been used for modifying nanoparticles of gold [12,13], silver [14,15], copper [16] and nickel [17]. Cysteine is also used in the synthesis or modification of nanoparticles which are then used as supports for enzymes. For example, Verma et al. [18] synthesized ZnO using L-cysteine, and then immobilized urease on the cysteine/ZnO nanoparticles. Results have also been reported concerning the modification of silica with cysteine and the use of the obtained material for immobilization of lipase [19]. Magnetic nanoparticles have also been functionalized with *Cys* and used as a support for xylose reductase [20]. Bezbradica et al. [21] prepared a matrix by chemical activation with cysteine and glutaraldehyde. The proposed support was used to immobilize four different molecules (trypsin, penicillin acylase G, lipase, and *E. coli* BL21 cell extract). In these studies, the immobilization is promoted through a two-step mechanism: in a first step, the enzyme is adsorbed on the support via an anionic exchange mechanism and, then, the covalent immobilization occurs. Immobilization on standard amino support activated with glutaraldehyde is usually via a first ionic exchange, then the covalent bonds may be produced. This is, in fact, a heterofunctional support [22,23]. In most cases, the imine linkage is formulated between glutaraldehyde-activated support and amino groups of enzyme, which should be later reduced to strengthen the linkage. However, most of the proteins are immobilized at neutral pH on glutaraldehyde-activated supports because imine in aqueous medium is unstable and the equilibrium enzyme support is shifted to the dissociated form. According to this, the linkages on glutaraldehyde-activated supports are performed through the reaction with cycled forms of the glutaraldehyde. This may cause that linkages are more stable than the imines [24,25]. Additionally, the surface of the support functionalized with cysteine is positively and negatively charged. If that surface is activated with glutaraldehyde, the mixed anionic/cationic exchange between enzyme and support takes place [26]. However, it also should be mentioned that, if the support has primary amino groups, and is modified with glutaraldehyde, the covalent bonds can also take place [27,28].

Dyes are organic, colored compounds which are capable of dyeing animal fibers (wool, silk), plant fibers (cotton, flax), leather, etc. The color of organic dyes depends on the presence in the molecule of chromophores (responsible for color formation) and auxochromes (electron donors which also increase the color by improving the solubility and adhesion of the dye to the fiber) [29,30]. Organic dyes are classified as chemical (e.g., nitro, anthraquinone, indigoid) and technical (e.g., acid, basic, vat, and reactive) [31]. Organic dyes are among the most significant contaminants of wastewater, because of their extensive use in numerous industries [32]. Alizarin Red S (ARS) is a 3-substituted derivative of 1,2-dihydroxy-9,10-anthraquinone, which belongs to the group of most durable dyes in textile wastewaters. That dye is water soluble and has application in histological studies to identifying calcium in tissues and vital staining of bone. The ARS can induce structural and functional changes to serum albumins [33]. The research show that ARS are also introduced adverse effects to organisms, such as oxidative damages. Moreover, other experimental show that anthraquinone and its sulfonated

derivatives could cause cytotoxicity, genotoxicity and DNA strand breakage [34]. Many methods are used for the degradation and decolorization of dyes. These methods can be divided into three categories: physical methods (nano-filtration, reverse osmosis, electrodialysis) [35], sorption techniques (photochemical, electrochemical destruction) [36] and biological methods (enzymatic degradation) [37]. Biological methods have low running costs, produce stable and harmless final products, and also require fewer chemicals and less energy than physical and chemical methods. Furthermore, enzymatic degradation complies with the principles of green technology [38]. Immobilized enzymes, especially oxidoreductases (laccases and peroxidases), are used to improve decolorization methods. Many dyes—for example, Acid Blue, Reactive Blue, Remazol Brilliant R, Direct Red etc.—have been decolorized using immobilized laccase. For this purpose, MOFs, bacterial nanocellulose, electrospun fibers and graphene oxide have been utilized as supports for the immobilization of laccase [39–42]. Besides the laccase, the different peroxidases were also successfully used to decolorize organic dye-based wastewaters [43–45].

In this study, we propose a novel cysteine-functionalized M_xO_y material as a support for enzyme immobilization. The materials used are SiO_2 and ZrO_2, prepared by the sol-gel method. Cysteine was applied in situ, that is, during the sol-gel synthesis. Additionally, the obtained material was activated with glutaraldehyde to improve the attachment of laccase to the material surface. Next, laccase from *Trametes versicolor* (light, brown powder with activity above 0.5 U/mg) was immobilized on the cysteine-functionalized M_xO_y by a simple adsorption method. The research included evaluation of physicochemical properties (Fourier-transform infrared spectroscopy, thermogravimetric, porous structure, zeta potential and elemental analysis) and catalytic properties (relative activity, kinetic parameters, influence of pH, temperature, storage and reuse on enzymatic activity). The obtained biocatalytic system was also tested in the decolorization of an organic dye (Alizarin Red S).

2. Materials and Methods

2.1. Materials

Sol-gel method: tetraethyl orthosilicate (TEOS), zirconium isopropoxide (ZIP), NH_{3aq} (25%), ethanol, isopropanol, L-cysteine (*Cys*), HCl. Immobilization process: glutaraldehyde (GA), laccase from *Trametes versicolor* (Lac), buffers: acetate (0.1 M; pH = 2–5), phosphate (0.1 M; pH = 6–8) and TRIS (0.1 M; pH = 9–10), Bradford reagent, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Decolorization of dyes: Alizarin Red S (ARS). All materials were purchased from Sigma-Aldrich® (St. Louis, MO, USA).

2.2. Synthesis of L-Cysteine-Functionalized M_xO_y

The metal oxides (SiO_2 and ZrO_2) were synthesized by a sol-gel method. In the first stage, an appropriate alcohol (ethanol for SiO_2 and isopropanol for ZrO_2) was introduced into a reactor. Next, the organic precursor (TEOS for SiO_2 and ZIP for ZrO_2) and the promotor of hydrolysis ($NH_{3aq.}$) were dosed. Then L-cysteine (10% wt./wt., in 1 M of HCl) was added. The components were mixed for 1 h (at ambient temperature) and left to age for 48 h. The synthesized materials were dried at 105 °C for 12 h. Finally, the obtained powder was washed several times using distilled water and alcohol, and the prepared materials were again dried (12 h, 105 °C). As a result, the systems SiO_2_*Cys* and ZrO_2_*Cys* were obtained. The precise information regarding sol-gel synthesis is presented in Table 1.

Table 1. Details information about reagents composition used in sol-gel synthesis of M_xO_y system.

ZrO_2	SiO_2
Isopropanol—250 mL	Ethanol—100 mL
ZIP—60 mL	TEOS—17 mL
NH_{3aq}—30 mL	NH_{3aq}—11 mL

In the next step, SiO_2_*Cys* and ZrO_2_*Cys* were activated with glutaraldehyde (5% in pH = 7 buffer) for 24 h. This led to the systems SiO_2_*Cys*_GA and ZrO_2_*Cys*_GA.

The four kind of supports (SiO_2_*Cys*; SiO_2_*Cys*_GA, ZrO_2_*Cys* and ZrO_2_*Cys*_GA) were used in the next step of research.

2.3. Immobilization of Laccase from Trametes Versicolor and Bradford Analysis

Immobilization of laccase from *Trametes versicolor* was led by adsorption and covalent method. The support (0.5 g of SiO_2_*Cys*; SiO_2_*Cys*_GA; ZrO_2_*Cys* and ZrO_2_*Cys*_GA) was added to the laccase solution (25 mL of solution with the concentration of 5 mg/mL in 0.1 M buffer at pH = 4). The immobilization process took place for 3 h at 25 °C in an incubator (IKA-Werke, Staufen, Germany). Bradford analysis was used to calculate the quantity of immobilized enzyme [46]. The quantity of immobilized enzyme ($mg/g_{support}$) is determined from the difference between the initial amount of enzyme and the final laccase concentration in the mixture after immobilization. The calculation was made relative to the mass of the support. The quantity of immobilized laccase (P) and immobilization yield (IY) were calculated using Equations (1) and (2):

$$P = \frac{(C_0 - C_1) \times V}{m} \quad (1)$$

$$IY = \frac{C_1}{C_0} \times 100 \quad (2)$$

where C_0 and C_1 denote the concentration of the enzyme (mg/mL) in solution before and after immobilization, respectively, V is the volume of solution (mL), and m is the mass of support (g). The following four biocatalytic systems were prepared: SiO_2_*Cys*_Lac; SiO_2_*Cys*_GA_Lac; ZrO_2_*Cys*_Lac and ZrO_2_*Cys*_GA_Lac.

2.4. Physicochemical Characterization

Spectroscopic, thermogravimetric, porous structure, zeta potential and elemental analysis were applied to characterize the samples obtained during the study.

The pure supports (ZrO_2_*Cys*, ZrO_2_*Cys*_GA, SiO_2_*Cys* and SiO_2_*Cys*_GA) were evaluated by means of thermal and elemental analysis. Thermogravimetric analysis (TG/DTG) was performed using a Jupiter STA 449F3 thermogravimetric analyzer (Netzsch, Selb, Germany). A sample (ca. 5 mg) was heated in a nitrogen atmosphere in the temperature range 30–1000 °C.

Contents of carbon, nitrogen, hydrogen and sulfur were evaluated to confirm the effectiveness of modification with cysteine. For this purpose, the Vario EL Cube apparatus (Elementar Analysensysteme, Langenselbold, Germany) was used. The analyzed sample (ca. 20 mg) was combusted in an oxygen atmosphere. After passing through a reduction tube, the resulting gases were separated in an adsorption column, and then recorded using a detector. The results are given as averages of three measurements, each accurate to 0.0001%.

Other analyses were used to characterize samples obtained before and after immobilization. Spectroscopic analysis was performed based on FTIR spectra obtained using a Vertex 70 spectrometer (Bruker, Billerica, MA, USA). The analyzed sample had the form of a tablet, made by pressing a mixture of anhydrous KBr (ca. 0.25 g) and 0.001 g of the analyzed material in a special steel ring under a pressure of 10 MPa.

Basic porous structure parameters of prepared samples were determined using an ASAP 2020 instrument (Micromeritics Instrument Co., Norcross, GH, USA). Before the analysis, all samples were degassed (support at 120 °C and biocatalytic system at 70 °C) for 4 h prior to measurement. Next, based on low-temperature N_2 sorption the analysis was carried out. Using the BET (Brunauer–Emmett–Teller) and BJH (Barrett–Joyner–Halenda) methods, the surface area (A_{BET}), total pore volume (V_p) and mean pore diameter (S_p) were assessed. Due to the high accuracy of the

instrument used, surface area was determined to an accuracy of 0.1 m^2/g, pore volume to 0.01 cm^3/g, and pore size to 0.01 nm.

Additionally, the zeta potential and isoelectric point (IEP) were evaluated using the LDV (Laser Doppler Velocimetry) technique, and calculated based on the Henry equation. These parameters were determined using a Zeta Nano ZS equipped with an MPT-2 automatic titration system (Malvern Instruments Ltd., Malvern, Worcester, UK). For the measurement, 0.01 g of the sample was dispersed in 25 mL of sodium chloride solution. Titration was performed with 0.2 M solutions of HCl and NaOH. The standard deviation of the zeta potential measurement was 61.5 mV. The apparatus measures a single zeta potential 30 times at defined pH, and the average value is used as the final result. The standard deviation of the pH value measurement was 0.1.

2.5. Catalytic Properties

The influence of pH, temperature, storage stability and reusability on the catalytic activity of a biocatalytic system is the most important information describing an immobilized enzyme. The influence of temperature was tested in the range 30–70 °C, and the influence of pH in the range 3–7. Storage stability was evaluated after 30 days (the immobilized enzyme was stored in a pH = 4 buffer at 4 °C). Reusability is the most important parameter for an immobilized enzyme; thus, the relative activity was investigated after 10 cycles. For clearer presentation of the data, in these experiments, the highest activity of free and immobilized laccase was defined as 100% activity. All of the above parameters were determined based on the reaction of oxidation of ABTS. In this case, 10 mg of free or immobilized laccase was added to 20 mL of 0.1 mM ABTS. The reaction was carried out for 20 min at 40 °C. Next, the mixture was centrifuged, and the absorbance was measured at λ = 420 nm (V-750 spectrophotometer, Jasco, Oklahoma City, OK, USA). The required parameters to define the immobilization process were determined [47]. The apparent activity of laccase was defined as the quantity of enzyme which oxidized 1 μM of ABTS per minute per 1 g of support. The activity retention and specific activity of laccase immobilized on the support were calculated according to Equations (3) and (4):

$$A_R = \frac{A_{S1}}{A_{S0}} \times 100\% \quad (3)$$

$$A_S = \frac{A_{Ap}}{P} \quad (4)$$

where A_R—retention activity (%); A_S—specific activity (U/mg_{enzyme}); A_{S1}—specific activity of immobilized Lac (U/mg); A_{S0}—specific activity of free Lac (U/mg); A_{Ap}—apparent activity ($U/g_{support}$); P—amount of immobilized Lac (mg/g).

Additionally, the kinetic parameters (K_M, the Michaelis-Menten constant; and V_{max}, the maximum reaction rate) were evaluated based on the above-mentioned ABTS oxidation reaction and calculated using Hanes–Woolf plots. In this process, various concentrations of the ABTS solution (0.005–1.5 M) were used.

All measurements were made in triplicate. Results are presented as mean ± 3.0 SD.

2.6. Decolorization of Alizarin Red S

A process of decolorization of Alizarin Red S dye was carried out using the four prepared biocatalytic systems. For this purpose, 100 mg of each of the biocatalytic systems was placed in 10 mL of Alizarin Red S dye solution (50 mg/mL; pH = 7). The process was performed at 30 °C for 24 h. The influence of time (0.5, 1, 3, 6, 9, 12, 24 h), temperature (25–70 °C) and the pH of the environment (2–9) on the effect of decolorization of the dye was determined. During these tests, differences in pH in absence of enzyme did not influence dye decolorization. Each experiment was carried out in triplicate, and the results are presented as average values.

After each of the above-mentioned experiments the absorbance of the resulting solution was measured (α = 464 nm; V-750 spectrophotometer, Jasco, Oklahoma City, OK, USA). The efficiency of decolorization of the dye was calculated based on the value of absorbance and using Equation (5):

$$ED = \frac{C_B - C_A}{C_B} \times 100\% \quad (5)$$

where ED is the efficiency of decolorization of ARS; C_B and C_A are the concentrations of ARS dye before and after the decolorization process, respectively.

3. Results

3.1. Thermogravimetric and Elemental Analysis of Pure Supports (ZrO_2_Cys, ZrO_2_Cys_GA, SiO_2_Cys and SiO_2_Cys_GA)

The materials used as supports for the immobilization of enzymes should have high thermal stability. Thermogravimetric analysis is one of the methods of evaluating the thermal stability of materials. The TG/DTG curves of pure cysteine show that this material has low thermal stability (Figure 1a,b). Two mass losses are observed: the first at ca. 200 °C related to physically adsorbed water, and the second at ca. 400 °C corresponding to the release of crystallization water. Zirconium and silica oxides offer good thermal stability [48]. As shown in Figure 1, all of the materials prepared in this study have high thermal stability. A mass loss of 20% was observed over the whole analyzed temperature range (30–1000 °C) for ZrO_2_*Cys* (Figure 1a). An additional mass loss at 900 °C was observed for the ZrO_2_*Cys*_GA system, probably associated with the thermal decomposition of glutaraldehyde. For SiO_2_*Cys* and SiO_2_*Cys*_GA, the mass loss was smaller than in the case of the zirconium materials. In this case the total mass loss was 10% over the analyzed range of temperatures (30–1000 °C). To summarize these results, it was found that the proposed cysteine-functionalized ZrO_2 or SiO_2 materials are suitable for use as supports in the immobilization of enzymes.

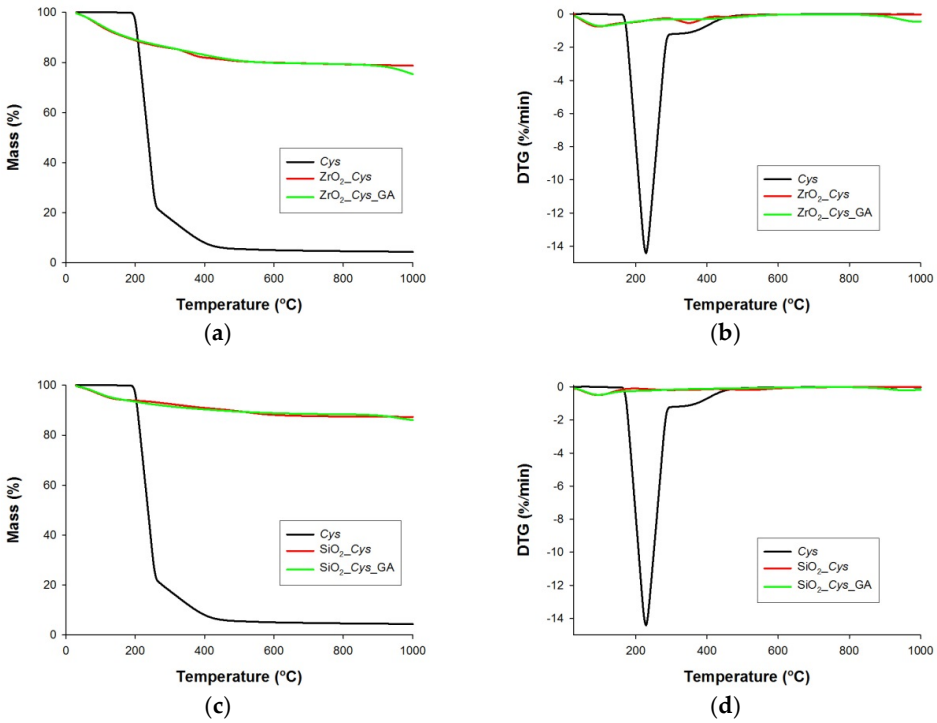

Figure 1. Thermogravimetric curves and their first derivatives (DTG). (**a**,**c**) ZrO_2_*Cys* and ZrO_2_*Cys*_GA; (**b**,**d**) SiO_2_*Cys* and SiO_2_*Cys*_GA.

Moreover, contents of N, C, H and S were determined to confirm the functionalization with cysteine and glutaraldehyde. The results are given in Table 2. To confirm the effectiveness of the modification, the table also provides data for pure ZrO_2 and SiO_2. An analysis of these data shows that the modification with cysteine and glutaraldehyde was successful. The contents of N, C and S increased for the sample with *Cys* and GA. The systems ZrO_2_*Cys* and ZrO_2_*Cys*_GA have higher contents of nitrogen, carbon and sulfur (N = 0.49%, C = 3.32% and S = 2.18%; N = 0.49%, C = 4.46% and S = 2.14%, respectively) than SiO_2_*Cys* and SiO_2_*Cys*_GA (N = 0.07%, C = 1.01% and S = 0.12%; N = 0.09%, C = 1.57% and S = 0.11%, respectively). These results show that functionalization with *Cys* and GA was more effective for ZrO_2-based samples.

Table 2. Content of N, C, H and S in the analyzed samples.

Sample Name	N	C	H	S
	(%)			
ZrO_2	0.05	1.09	2.60	0.15
ZrO_2_*Cys*	0.49	3.32	2.63	2.18
ZrO_2_*Cys*_GA	0.49	4.46	2.61	2.14
SiO_2	0.04	0.15	1.67	0.08
SiO_2_*Cys*	0.07	1.01	1.68	0.12
SiO_2_*Cys*_GA	0.09	1.57	1.40	0.11

3.2. Spectroscopic, Porous and Zeta Potential Analysis of Samples before and after Immobilization

FTIR analysis is one of the most common methods used to identify the characteristic groups present in samples. In this study, samples were evaluated by this method both before and after immobilization. The FTIR spectra are shown in Figure 2, and the characteristic groups are summarized in Table 3. The amino (N–H; 3215 cm^{-1}), thiol (S–H; 2550 cm^{-1}) and carboxyl (C–O; 1585 cm^{-1}) groups are observed in cysteine (Figure 2a,c). Moreover, ZrO_2-*Cys* and ZrO_2_*Cys*_GA contain several groups appearing at 3180–3650 (N–H and O–H), 1610 (C–O), under 1000 (Zr–O, Zr–OH and Zr–O–Zr) and 2845 cm^{-1} (C–H; only for the sample with GA) (Figure 2a). Furthermore, N–H (3450 cm^{-1}), C–H (2960 cm^{-1}) and amide I, II and III (1620, 1480 and 1320 cm^{-1}, respectively) groups are present in the enzyme structure (Figure 2b,d). The systems with laccase immobilized on ZrO_2_*Cys* and ZrO_2_*Cys*_GA contain groups characteristic of both the support and free laccase. The most characteristic bands on the FTIR spectrum of ZrO_2_*Cys*_Lac appear at 1615, 1492 and 1334 cm^{-1}, corresponding to amide I, II and III, respectively. However, ZrO_2_*Cys*_GA_Lac contains only amide I and III (1616 and 1328 cm^{-1}). A similar situation is found for the SiO_2-based materials. Signals for N–H and O–H (3176–3578 cm^{-1}), C–O (1622 cm^{-1}), Si–O, Si–O–Si (>1000 cm^{-1}) and C–H (2875 cm^{-1}) can be identified on the spectra of SiO_2_*Cys* and SiO_2_*Cys*_GA. However, the SiO_2_*Cys*_Lac and SiO_2_*Cys*_GA_Lac spectra contain, besides the characteristic groups for the support, only a small peak at 1624 cm^{-1} corresponding to the amide I band. Based on the FTIR results, the effectiveness of immobilization of laccase can be partially confirmed. Changes in the range 1650–1310 cm^{-1} (corresponding to amide I, II and III) confirmed the presence of laccase molecules on the cysteine-functionalized zirconia and silica materials.

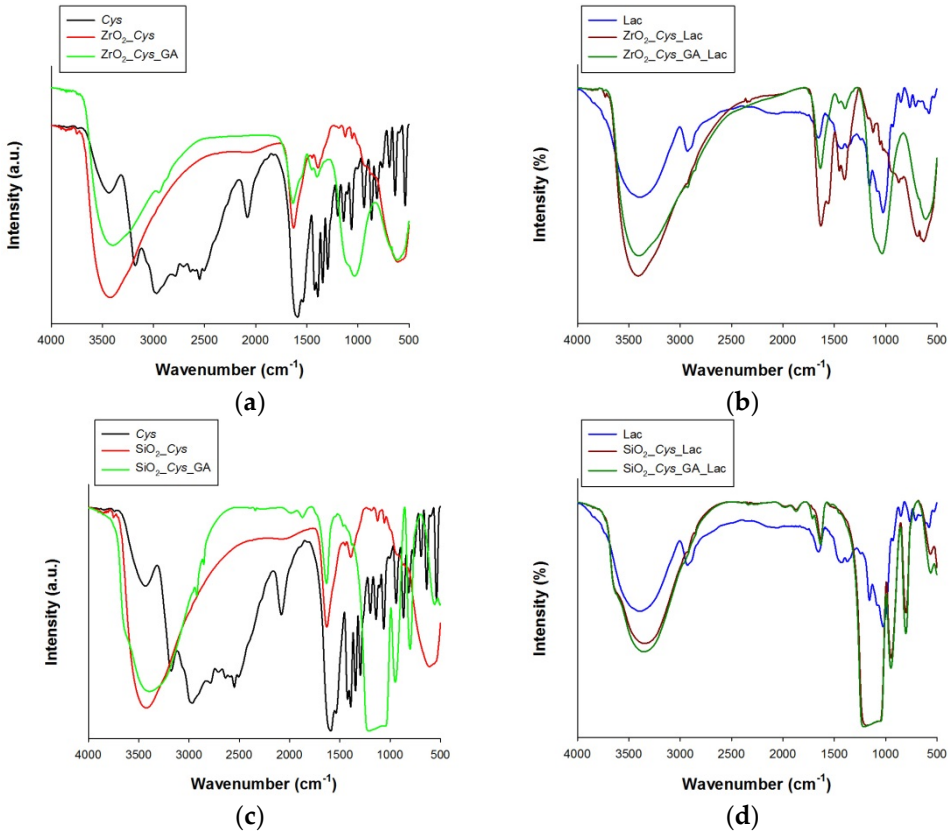

Figure 2. FTIR spectra of samples before (**a**,**c**) and after (**b**,**d**) immobilization of laccase.

Table 3. Characteristic groups present in cysteine, laccase, and samples before and after immobilization of laccase.

Sample	Characteristic Groups	Wavenumber (cm^{-1})
Cys	N–H	3215
	S–H	2550
	C–O	1585
Lac	N–H	3450
	C–H	2960
	Amide I, II and III	1620, 1480 and 1320
ZrO_2_*Cys*	N–H, O–H	3180–3650
	C–O	1610
	Zr–O, Zr–O–Zr	>1000
ZrO_2_*Cys*_GA	C–H	2845
ZrO_2_*Cys*	Amide I, II and III	1615, 1492 and 1334
ZrO_2_*Cys*_GA	Amide I and III	1616 and 1328
SiO_2_*Cys*	N–H, O–H	3176–3578
	C–O	1622
	Si–O, Si–O–Si	>1000
SiO_2_*Cys*_GA	C–H	2875
SiO_2_*Cys*_Lac	Amide I	1624
SiO_2_*Cys*_GA_Lac		

The porosity of materials also plays an important role in enzyme immobilization. A classical method of immobilization is adsorption. Materials may be potential supports if they have a well-developed porous structure. In Table 4, the basic porous parameters (surface area, maximum pore volume and

mean pore diameter) are shown. The ZrO_2_*Cys* and ZrO_2_*Cys*_GA systems have large surface areas of 295 and 262 m^2/g, respectively. After immobilization the surface area decreased, attaining values of 292 m^2/g for ZrO_2_*Cys*_Lac and 146 m^2/g for ZrO_2_*Cys*_GA_Lac. This is probably associated with the attachment of laccase to the support surface and the blocking of pores. The SiO_2-based materials (SiO_2_*Cys* and SiO_2_*Cys*_GA) have smaller surface areas (20 and 17 m^2/g) than the ZrO_2-based materials. However, the surface area again decreased after immobilization. In this case, the pore diameter was also reduced after immobilization, which may be related to the deposition of laccase inside the pores.

Table 4. Porous parameters of samples before and after immobilization of laccase.

Sample	A_{BET} (m^2/g)	V_p (mL/g)	S_p (nm)
ZrO_2_*Cys*	295	0.032	1.9
ZrO_2_*Cys*_Lac	292	0.034	1.9
ZrO_2_*Cys*_GA	262	0.029	1.9
ZrO_2_*Cys*_GA_Lac	146	0.026	1.9
SiO_2_*Cys*	20	0.018	4.2
SiO_2_*Cys*_Lac	16	0.007	2.1
SiO_2_*Cys*_GA	17	0.006	2
SiO_2_*Cys*_GA_Lac	13	0.006	2.1

In the final part of the physicochemical analysis, the zeta potential (ζ) and isoelectric point (IEP) were determined (Figure 3). Based on the zeta potential values, the electrostatic interactions between the enzyme and support were investigated. The value of ζ for ZrO_2_*Cys* at all analyzed pH (2–10) is between 40 and –50 mV, with the isoelectric point at 4.57 (Figure 3a). Small changes in the zeta potential are observed after the immobilization of laccase on ZrO_2_*Cys* (ZrO_2_*Cys*_Lac), and the isoelectric point of ZrO_2_*Cys*_Lac occurs at pH = 4.00. Analyzing the zeta potential and isoelectric point values of ZrO_2_*Cys*_GA (before and after immobilization) no significant changes were observed (Figure 3b). A similar situation was found for the samples based on SiO_2, before and after immobilization of laccase. However, the zeta potential of the proposed biocatalytic systems is more negative. This is associated with the slipping plane, which is slightly stronger after the immobilization of laccase onto cysteine-functionalized ZrO_2 and SiO_2 materials [49,50].

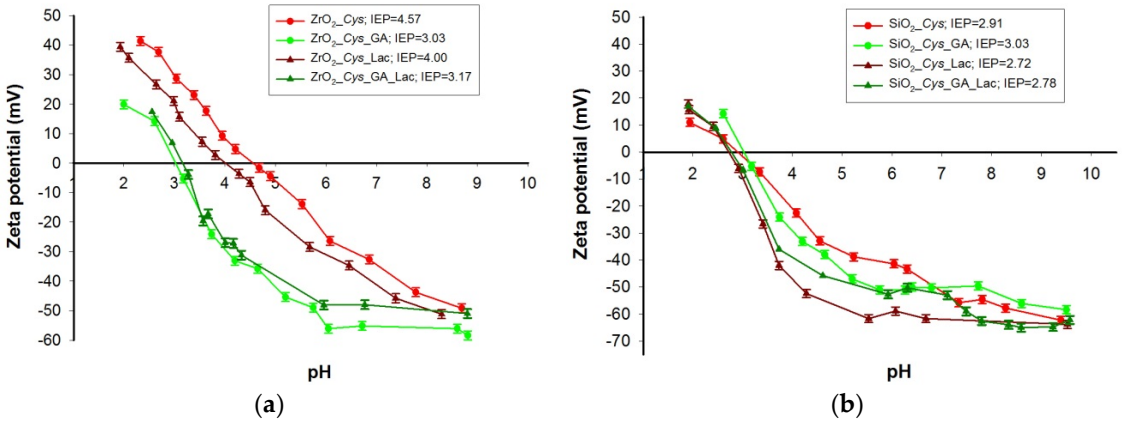

Figure 3. Zeta potential values as a function of pH for samples based on (**a**) ZrO_2 and (**b**) SiO_2 before and after immobilization.

Based on the FTIR, BET and potential zeta results, a mechanism for the immobilization of laccase on cysteine-functionalized M_xO_y (activated or not with glutaraldehyde) was proposed and presented in Figure 4. The results from FTIR and zeta potential analysis suggest that the immobilization of laccase

on M_xO_y_*Cys* without glutaraldehyde take place through non-specific forces such as Van der Waals and electrostatic interactions. Whereas, the activation of M_xO_y_*Cys* with glutaraldehyde is usually via a first ionic exchange. Then the immobilization by covalent bonds is possible. In addition, the surface of cysteine-functionalized M_xO_y is positively and negatively charged. When that surface is activated with glutaraldehyde, the mixed anion/cationic exchange takes place [26]. On the other hand, the changes in porous structure observed after immobilization suggest that enzyme was adsorbed inside the pores of support.

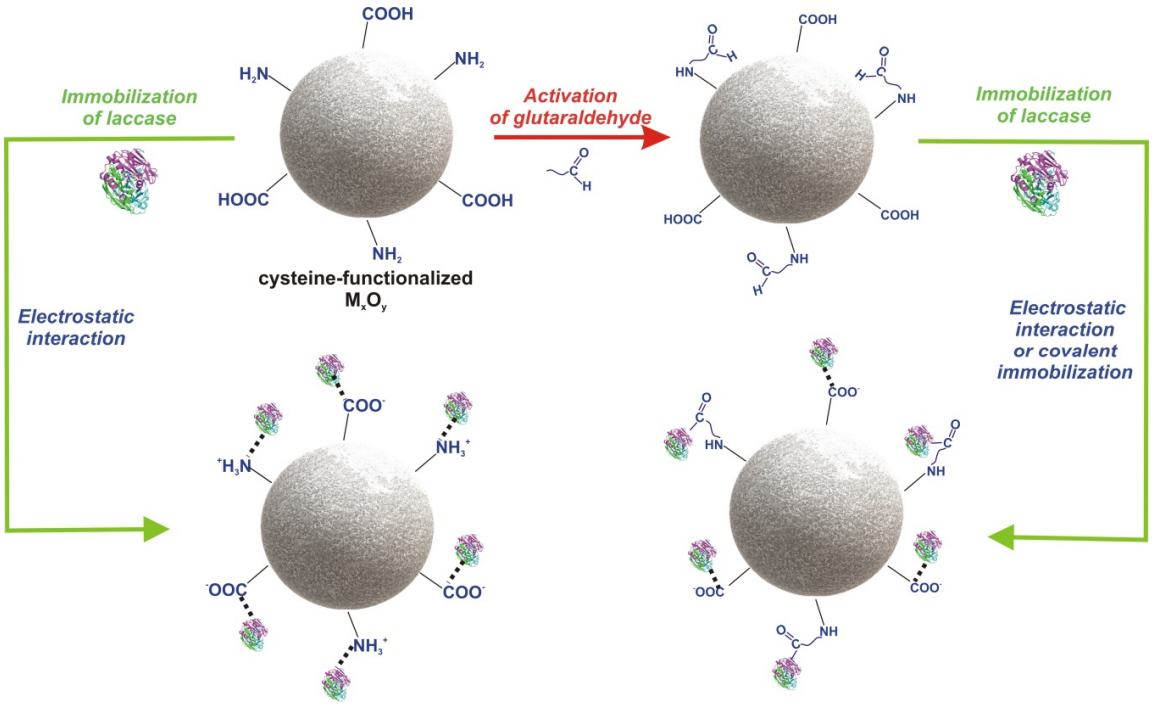

Figure 4. A suggested mechanism of immobilization of laccase onto cysteine-functionalized M_xO_y.

3.3. Catalytic Properties of the Obtained Biocatalytic Systems

Catalytic parameters of the prepared biocatalytic systems are presented in Table 5. These data show that the highest quantity of laccase was immobilized on ZrO_2_*Cys* (250 mg per 1 g of support), while for the other systems the quantities of immobilized enzyme were smaller (212–225 mg/g). In all cases, the immobilization yield is in the range 94–99%. The activity of biocatalytic systems determines their possible applications. The specific activity of the ZrO_2_*Cys*_Lac biocatalytic system was found to be 19.3 U/g, compared with 25 U/mg for free laccase. This means that the immobilized laccase (ZrO_2_*Cys*_Lac) retained about 77% of the initial activity. The system ZrO_2_*Cys*_GA_Lac had slightly lower specific activity (13.5 U/mg) and activity retention (ca. 54%). The activity of laccase immobilized on ZrO_2_*Cys*_GA is lower than in the case of ZrO_2_*Cys*, because the modification with glutaraldehyde has an impact on both the enzyme structure and the enzymatic activity. For the other biocatalytic systems, significantly smaller values were obtained (0.6 U/mg and 2.6% for SiO_2_*Cys*_Lac; 7.1 U/mg and 28.5% for SiO_2_*Cys*_GA_Lac). Table 5 also contains kinetic parameters of the obtained biocatalytic systems, which indicate the affinity of laccase to its substrate. The Michaelis constant (K_M) for the laccase immobilized on ZrO_2-based materials (0.11–0.14 mM) is smaller than the value for free laccase (0.18 mM). Changes are also observed for the maximum retention velocity (V_{max}). The V_{max} values for laccase immobilized on cysteine-functionalized ZrO_2 were higher (0.095 mM/s for ZrO_2_*Cys*_Lac; 0.031 mM/s for ZrO_2_*Cys*_GA_Lac) than the value for free laccase (0.027 mM/s). These results indicate a slightly higher substrate affinity in the case of the immobilized biomolecules. The kinetic parameters of SiO_2_*Cys*_Lac and SiO_2_*Cys*_GA_Lac are similar to those of the free enzyme. Similar results

were obtained by Qiu et al. [51], which immobilized laccase onto inorganic mesoporous silica and natural organic polymer like chitosan using functional ionic liquid as bridging agent (SBA-CIL-CS). Kinetic parameters measurement showed that the SBA-CIL-CS-Lac had the outstanding affinity to the substrate.

The values of catalytic properties show that the kinetic parameters are changed after the immobilization process. The changes in kinetic parameters are caused by the transformations of the protein structure and the immobilization methods. Furthermore, a decrease in K_M leads to an increase in the enzyme's affinity to the substrate. This probably occurs when the electric charges on the support and substrate are of different sign [52,53].

Table 5. Catalytic and kinetic parameters describing the biocatalytic systems.

Sample	*P* (mg/g)	*IY* (%)	A_S (U/mg)	A_R (%)	K_M (mM)	V_{max} (mM/s)
Free Lac	-	-	25 ± 1.6	-	0.18 ± 0.024	0.027 ± 0.012
ZrO_2_*Cys*_Lac	250 ± 5.6	99.9 ± 0.7	19.3 ± 1.5	77.2 ± 2.8	0.11 ± 0.021	0.095 ± 0.011
ZrO_2_*Cys*_GA_Lac	225 ± 5.4	97.5 ± 0.7	13.5 ± 1.4	53.8 ± 2.5	0.14 ± 0.022	0.031 ± 0.013
SiO_2_*Cys*_Lac	216 ± 5.3	95.6 ± 0.6	0.6 ± 0.3	2.6 ± 0.8	0.17 ± 0.023	0.028 ± 0.010
SiO_2_*Cys*_GA_Lac	212 ± 5.3	94.5 ± 0.6	7.1 ± 1.1	28.5 ± 1.9	0.18 ± 0.022	0.029 ± 0.011

The immobilized enzymes are less sensitive to pH and temperature changes, retain high activity after many days of storage, and can be used in several reaction cycles. Figure 5 shows the influence of pH, temperature, storage stability and reusability on the activity of free and immobilized laccase. All of the biocatalytic systems obtained in this study retain good activity under various chemical and thermal conditions. Furthermore, good activity is retained after a number of days of storage and after several reaction cycles. The ZrO_2_*Cys*_Lac and ZrO_2_*Cys*_GA_Lac systems maintain residual activity above 40% at all analyzed pH and temperature values. Furthermore, ZrO_2_*Cys*_Lac retains high (above 90%) activity after 30 days of storage and after 10 reaction cycles. Laccase immobilized on SiO_2_*Cys*_GA exhibits slightly lower activity (above 60%) in the same conditions. It preserves about 20–30% of its initial activity at pH = 3–7 and ca. 40% at temperatures of 30–70 °C. Its activity after 30 days of storage and after 10 reaction cycles is only 30–40% and 50–60%, respectively.

Table 6 summarizes the above results together with the results of other studies in which different cysteine-functionalized supports were used to immobilize various enzymes. The table shows that the results obtained using cysteine-functionalized ZrO_2 and SiO_2 materials as supports for laccase are satisfactory compared with the other results. As shown, cysteine has been used to modify Ag, Cu, ZnO and poly(glycidyl methacrylate)-SiO_2, utilized as supports for alkaline phosphatase, urease and lipase, respectively [15,16,18,19]. Upadhyay et al. [15] proposed a cysteine-Ag/AP biocatalytic system, which exhibited a specific activity of 6.31 U/mg and activity retention of 67%, and retained 60% of its initial activity after seven reaction cycles. Kumar et al. [16] and Verma et al. [18] immobilized urease on cysteine-Cu and cysteine-ZnO, respectively. In both cases the activity retention was ca. 72%. Good results were also obtained by Chen et al. [19], who prepared cysteine-poly(glycidyl methacrylate)-SiO_2/Lip biocatalysts with high specific activity (44.1 U/mg) and activity retention (63.3%). That system was also used over eight reaction cycles, retaining 40% of its activity.

Summarizing performed research it has been shown, that the catalytic properties of enzyme in different pH, temperature, storage stability and reusability were improved. The support stabilizes and stiffness the enzyme structure, which consequently protect enzyme against denaturation in extreme pH and temperature conditions. Moreover, the carrier exposes the active sites of the catalyst for easy attachment of substrate molecules and reduce diffusional resistance of the substrates and products. The best catalytic properties were obtained when laccase was immobilized onto ZrO_2_*Cys*. It can be associated with the properties of that materials, especially well-developed porous structure. Furthermore, the data from elemental analysis showed that the cysteine was successfully introduced into the ZrO_2. The activation with glutaraldehyde causes lowering of catalytic activity. It may be

related with the presence of covalent bonds, which can block active site of enzyme and in consequently reduced enzymatic activity.

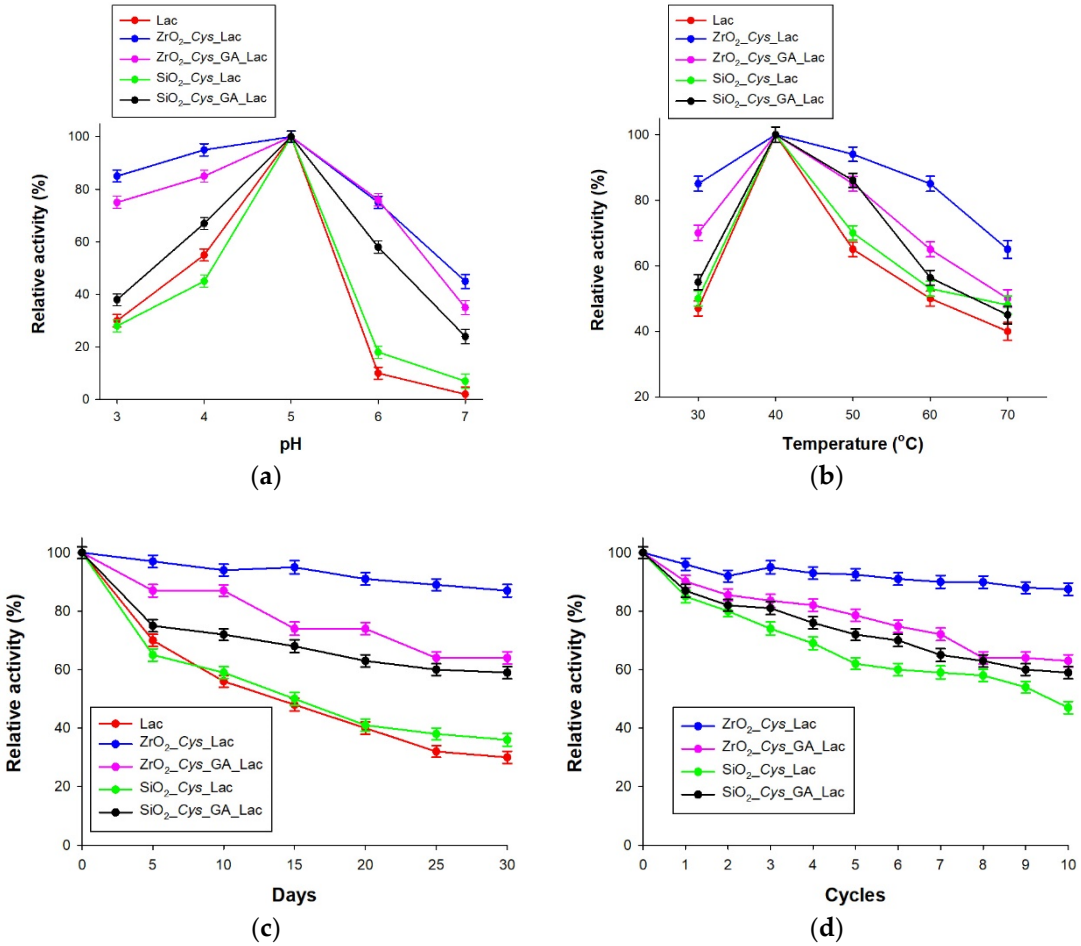

Figure 5. Influence of (**a**) pH, (**b**) temperature, (**c**) storage stability and (**d**) reusability on relative activity of free and immobilized laccase.

Table 6. Summary of catalytic properties obtained for different enzymes immobilized on cysteine-functionalized materials.

Support	Enzyme	*As* (U/mg)	A_R (%)	Number of Cycles; Residual Activity (%)	References
cysteine-Ag nanoparticles	alkaline phosphatase (AP)	6.31	67	7; >60	[15]
cysteine-Cu	urease (Ur)	45.8	72.4	10; >80	[16]
cysteine-ZnO	urease (Ur)	3.42	72.5	-	[18]
cysteine-poly(glycidyl methacrylate)-SiO_2	lipase (Lip)	44.1	63.3	8; >40	[19]
cysteine-ZrO_2	laccase (Lac)	19.3	77.2	10; >90	This study
cysteine-ZrO_2-glutaraldehyde	laccase (Lac)	13.5	53.8	10; >60	This study

3.4. Decolorization of Alizarin Red S

Laccase is an enzyme which is capable of degrading and decolorizing organic dyes from wastewaters. In this study, the obtained biocatalytic systems were used for the decolorization

of Alizarin Red S (ARS) dye. The results are presented in Figure 6, including the effect of time, pH and temperature. As shown in Figure 6a, the ZrO_2-based biocatalytic systems (ZrO_2_*Cys*_Lac and ZrO_2_*Cys*_GA_Lac) produced a higher efficiency of decolorization of ARS dye over the analyzed process duration, as compared with the SiO_2-based biocatalytic systems (SiO_2_*Cys*_Lac and SiO_2_*Cys*_GA_Lac). The highest decolorization efficiency was achieved after 24 h (95% for ZrO_2_Cys_Lac and 85% for ZrO_2_*Cys*_GA_Lac), but an efficiency of ca. 90% was already reached after 5 h. After the same time, the efficiency of decolorization of ARS using SiO_2_*Cys*_Lac and SiO_2_*Cys*_GA_Lac reached 35% and 20%, respectively. Laccase immobilized on ZrO_2-based materials results in higher decolorization of ARS dye because of the high specific activity and activity retention of these systems. It was observed that Alizarin Red S can be successfully decolorized by immobilizing laccase on ZrO_2-based materials. Furthermore, the efficiency of decolorization of Alizarin Red S in this study was higher than in previous reports where immobilized laccase was used. Zhao et al. [54] immobilized laccase on mesostructured cellular foam silica (MCF), and obtained maximum decolorization of Alizarin Red S equal to 73%. Similarly, Lu et al. [55] used alginate–chitosan microcapsules as a support for laccase. That biocatalytic system was utilized in the decolorization of ARS, and the efficiency of decolorization was measured at 70%.

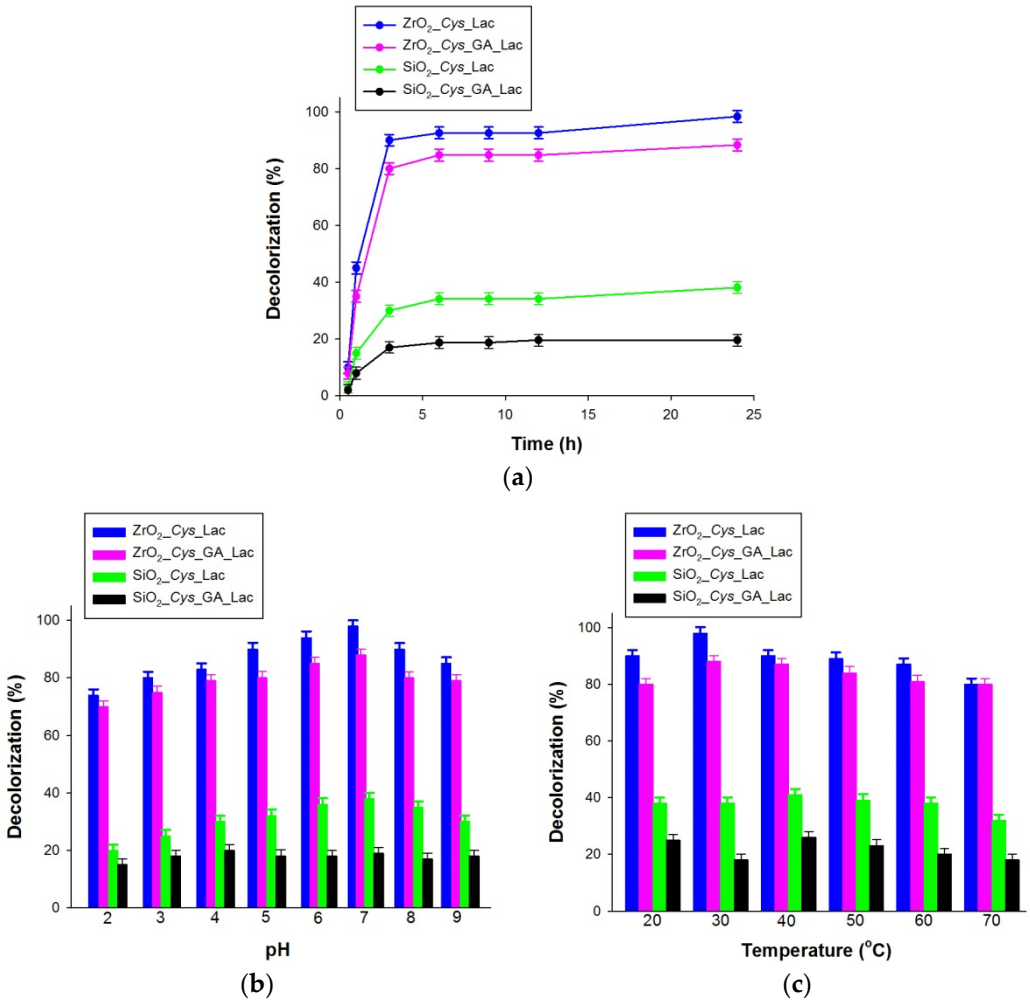

Figure 6. Effect of (**a**) process duration, (**b**) pH and (**c**) temperature on the efficiency of decolorization of Alizarin Red S.

The immobilization process serves to produce biocatalytic systems that can be used in a range of reaction conditions. Therefore, decolorization was carried out under different pH and temperature

conditions (Figure 6b,c). Laccase immobilized on ZrO_2_*Cys* and ZrO_2_*Cys*_GA achieved a high efficiency of decolorization (above 70%) in the whole of the analyzed pH and temperature ranges. Laccase immobilized on SiO_2_*Cys* and SiO_2_*Cys*_GA resulted in significantly lower efficiency of decolorization of ARS dye, reaching at most 40%. The results indicate that the biocatalytic systems proposed in this study (especially ZrO_2_*Cys*_Lac and ZrO_2_*Cys*_Ga_Lac) have potential applications in the decolorization of dyes from wastewaters.

4. Conclusions

The experiments performed in this study showed that large quantities of enzyme were attached to the proposed materials. Cysteine-functionalized ZrO_2 produced a higher immobilization yield and enzymatic activity than cysteine-functionalized SiO_2, which may suggest the superior ability of ZrO_2-based materials to attach to enzymes. Good thermal and electrokinetic stability, a well-developed porous structure and the presence of specific groups enable the use of M_xO_y-based materials for the immobilization of laccase from *Trametes versicolor*. In addition, based on that analysis the catalytic (A_S and A_R) and kinetic (K_M and V_{max}) parameters of the obtained biocatalytic systems were determined based on the oxidation of ABTS. The values of these parameters indicate the higher affinity of immobilized laccase to the substrate compared with the free enzyme. Moreover, laccase immobilized on ZrO_2-based support retains high relative activity over a wide range of pH (>40%) and temperature (>50%), and also after 30 days of storage (>60%) and 10 reaction cycles (>60%). Most importantly, very good results were achieved in the decolorization of Alizarin Red S. In these tests, a high efficiency of decolorization (97%) was obtained. The biocatalytic systems proposed in this study, based on cysteine-functionalized metal oxide, may be used in removing other organic and pharmaceutical pollutants from wastewaters.

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