ORIGINAL RESEARCH PAPER

Removal of Malachite Green by Using Immobilized Glucose Oxidase Onto Silica Nanostructure-Coated Silver Metal-Foam

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ABSTRACT
Enzymes Immobilization onto different types of the substrate could be helpful in various applications of biomedical devices and biosensors. Enzyme activity and stability could be affected by support and method of immobilization. In this study, the silver metal foam was successfully synthesized by the soft-shell method and then was coated with silica. Then, glucose oxidase (GOx) immobilized on non-coated, and silica-coated silver metal foam and removal of malachite green was investigated. Fourier Transform Infrared Spectroscopy (FT-IR) and scanning electron microscopy (SEM) results confirmed that the enzyme was attached to support surface. The maximum immobilized enzyme activity was about 118.980 U/grSupport at 40°C. The removal of malachite green showed the indirect relation with dye concentration confirmed by decolorization assay. The high activity, thermal stability, and reusability of immobilized glucose oxidase onto the silver metal foam in comparison to free enzyme introduce the capability of the biological system for removal of malachite green in the industry.

Keywords: *Enzyme immobilization; Glucose oxidase; Silver metal foam; Enzyme activity; Malachite green*

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INTRODUCTION

The immobilization of biomolecules such as enzymes, antibodies, peptides and nucleic acids onto an inorganic support is almost desirable for biotechnology and chemical applications [1]. The surface immobilization methods have been divided into four standard classes: (I) physical adsorption, where vanderwaals, electrostatic (Ionic bonds) and hydrophobic interactions, between support and enzyme exist [2-4] (II) chemical bonds, where covalent and coordinate bonds are shaped with the enzyme [5-8], (III) physical entrapment, [9, 10] and (IV) self-immobilization of enzyme aggregates or crystals using a bi-functional reagent to handle carrier-less macroparticles [11, 12].

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The stabilization of enzymes has been applied by using a different type of methods such as medium engineering, chemical crosslinking, and enzyme immobilization. A reported method to avoid the disruption of enzymes is to be capped their surface with ionic exchanges that might at once interact with several enzyme subunits, prohibiting enzyme disruption [13].

The Immobilization of protein onto a suitable support via ionic bond may give us greater stability [14, 15]. The macro particles, such as glass beads and sand [16], glass tubes surrounding the light source in the reactor [17] and some polymers [18, 19] could be employed as supports. A properly planned immobilization leads to an augmented rigidity of the heterogeneous adsorbed materials in biological systems. Therefore, the immobilization of enzymes has high promising potential on the improved stability, potential modulation of the catalytic properties, and aids to make easy the obstacle of microbial growth in certain cases [20, 21].

Control of stability and activity of enzymes significantly associated with the orientation of enzyme on the surface [22]. Furthermore, enhanced biocatalytic efficiency may be accomplished by the modified structure of the support. The immobilization of protein onto porous materials is one of the attractive methods which has been employed for the treatment of effluents [23]. Silver and silver porous mesh material, metal foam, and spongy material made from silver are preferably used for wastewater handling. Silver metal-foam (SMF) is able to be a useful support for enzyme immobilization because of having 93% porosity and perforations. The hole diameter from 0.2 to 1mm was used as a substrate [24, 25] and the silver-made expanded mesh also can be efficiently used in this case.

The surface modification is one of the significant methods for appropriate enzyme immobilization. Silica surfaces are able to modify the surface of the support by using diverse functional groups for suitable covalent bonding onto the enzymes. Therefore, the functionalized mesoporous silica materials are highly recommended for enzyme immobilization [26, 27].

Recently, the modern methods have been applied for oxidation and degradation of hazardous organic compounds due to the growth of these materials in the environment [28]. Malachite green (di[4-dimethylamino-phenyl] phenylcation) is a synthetic dye which has been employed in the textile industry to colour silk, wool, leather, cotton, and paper. Removal of the dyes from effluents in an economical style is a key problem for textile industries [29]. A number of processes have carried out for the handling of these pollutants which include biodegradation, advanced oxidation electrochemical oxidation such as anodic oxidation and electro-Fenton processes. Among these processes, oxidation processes have widely applied as the capable method for sewer and water handling [30-32].

The convenience of storage and handling in biotechnology process has been applied to achieve a successful simultaneous process, [33, 34]. Enzymes are extracted from different types of microorganisms such as fungi, bacteria, and insect, and they are functional biomolecules which are widely used in various biotechnology applications. Among the enzymes, glucose oxidase (GOx) is one of the most attractive enzymes producing in place hydrogen peroxide in the presence of glucose [28]. Hydrogen peroxide has extensively used as a substance with application in photochemical for treatment a basis of hydroxyl radicals. In situ method of H_2O_2 production can be fabricated one using sequential hydrogenation process, which itself generates considerable waste streams and devours large amounts of energy [35]. The enzymatic construction of hydrogen peroxide from GOx engaged a production method under gentle conditions for an alternative, localized hydroxyl radical with less environmental and cost implications [36]. The method is inexpensive and clean and prevents the danger of H_2O_2 related to storage, moving, and conducting of highly concentrated solutions. To the best of our knowledge, the immobilization of GOx enzyme onto SMF with silica-coated and uncoated has not been considered for removal of MG in wastewater.

In this work, SMF was used as a support surface for enzyme immobilization and then coated with silica nanostructure. After that, the effect of temperature on MG dye decolorization efficiency was investigated. In addition, the stability and reusability of GOx were studied, and different mechanisms of the photocatalytic–enzymatic process also were examined (Fig 1).

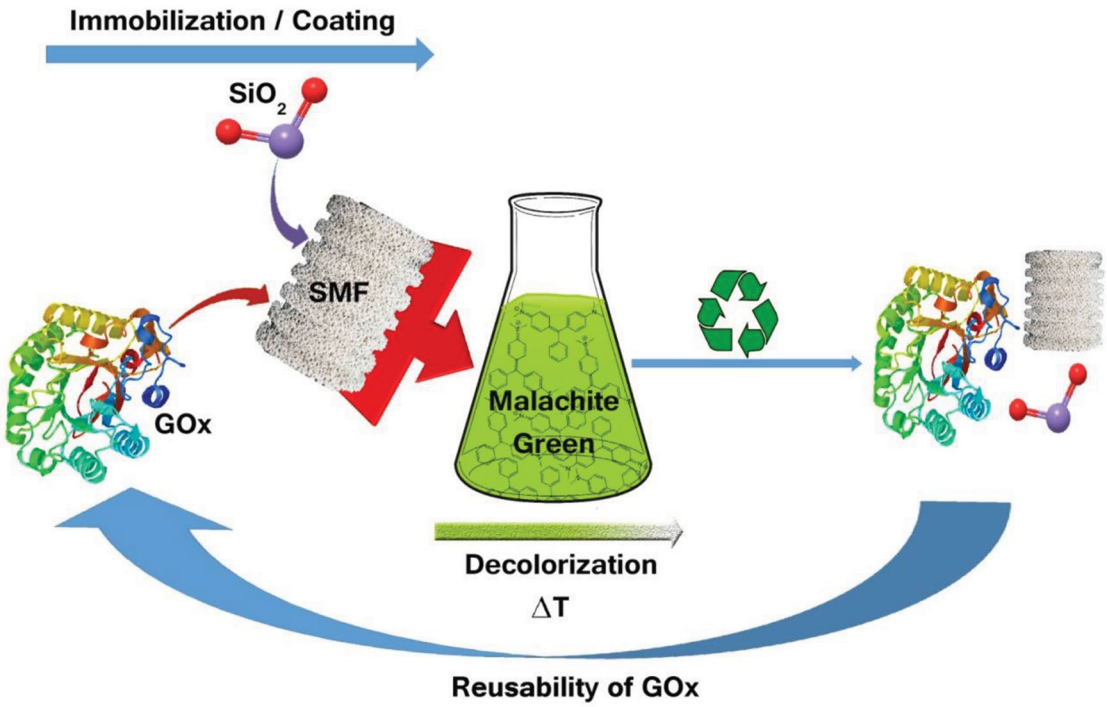

Fig. 1. Schematic conceptual design of removal of malachite green process by using immobilized Glucose oxidase onto silica-coated silver metal-foam.

MATERIAL AND METHODS

Materials

Industrial grade GOx (E.C.1.1.3.4) was purchased by Kimia Enzyme Company (Iran). Glucose was purchased from Sigma-Aldrich Company and has been prepared in distilled water as the substrate of GOx. The MG dye also purchases from Sigma-Aldrich. All other chemical materials purchased from Merck Millipore Co. (Germany) and used without future purifications.

Apparatus

The UV-Vis spectra were calculated by using a Shimadzu 1700-UV Spectrophotometer (Japan). The images were obtained by using MIRA3 FEG-SEM (Czech Republic) and Axio Star Plus (Japan). Physical evaporation deposit (PVD) was performed by the instrument from E-CAT (Russia).

Fabrication of Silver Metal Foam

The SMF was fabricated based on the soft-shell method by our company (Nano Pooshesh Felez). In this approach, the polyurethane foam in (10×10×5 cm) PVD coated with a thin layer of aluminum to be conductive. After that, the primary coating enriched by silver electrolysis bath and the latter, the coating thickness was increased by using the electroplating method until the desired strength is achieved. Lastly, the residual polyurethane foam is burnt for obtaining the pure SMF. The hollow structure of the SMF has shown a nano-metric topography at various scales.

Nano-structure Coating on Silver Metal Foam

The metal foams (2×2×1 cm) were submerged in 100ml of sodium silicate (industrial grade) which has been diluted in deionized water for 5min and then dried at 80°C. The procedure was repeated three times to obtain a suitable coating on the surface of the metal foam. Finally, metal-foams were heated in the oven (650°C) to calcinate the silica.

Characterization

FT-IR was applied by using KBr pellets to study functional groups of the fabricated SMF before and after immobilization. The morphology of the sample was examined by SEM.

Immobilization of GOx

According to Henderson-HasselBalch equation,

Acetate buffer (0.1M, pH: 5.5) was prepared by using acetic acid and sodium acetate. Phosphate buffers (0.1M, pH: 4.2-7) were made from sodium dihydrogen phosphate dihydrate and disodium phosphate and also developed based on the Henderson-HasselBalch method. The prepared buffers were used for immobilization of uncoated and coated SMF.

For GOx immobilization, 2.5gr enzyme dissolved in 25ml Phosphate buffer. The enzyme (0.1mg/ml) was filtered to achieve the maximum activity and stability. After that, the enzyme solution was added to the support and has been maintained in shaker-incubator for 0.5-4 h (15-47°C).

After immobilization, the supernatant removed and the support washed with acetate buffer to remove unbounded GOx. The amount of immobilized enzyme could be computed as follows:

Formula (1): (1-C/C_0)×V= immobilized Enz.

Where, V is the volume of the measured sample, C_0 and C is the concentration of GOx initially used for the reaction, and the unbound GOx collected in each purification cycle respectively.

GOx activity assay

The enzyme activity was measured by Colorimetric Method. The method is based on the production of Hydrogen peroxide by free and immobilized GOx in the presence of Oxygen and β-glucose. In this case, the obtained H_2O_2 (<1mM) measured by using the method based on absorption of produced I_3^- in the presence of I^- at 351nm (eq. 1, 2 and 3). The amount of producing peroxide is measured by I_3^- in the solution including 0.1M of phosphate buffer and 20mM of β-glucose.

Equation 1 $C_6H_{12}O_6+O_2+H_2O \xrightarrow{GOx} H_2O_2+C_6H_{12}O_7$

Equation 2 $H_2O_2+2I^- \xrightarrow{Mo(VI)} I_2+H_2O$

Equation 3 $I_2+I^- \rightarrow I_3^-$

Determination of thermal stability

Several samples of the immobilized and free enzyme were exposed to increasing temperatures for 1hr periods. The enzyme screen was assayed at two weeks intervals to determine loss enzyme activity with time (at 50°C). The samples were exposed at 30, 40, and 50°C. Then, 2.5gr enzyme was dissolved in 25ml phosphate buffer. The filtered enzyme (0.1mg/ml) was added to silica-coated SMF. The concentration of the free GOx was considered as the same of the immobilized enzyme in comparison.

Determination of reusability

The same method of enzyme assays (measuring of activity) was applied for the reusability. After each stage, the immobilized GOx were washed with DW to remove residual substrate and product before the subsequent experiment.

Decolorization measurement

In case of decolorization test, 12.5mL of enzyme solution (230 U/mL) with 0.1mg/mL concentration was mixed in 0.1M phosphate buffer (pH: 6.15) at 37°C in shaker-incubator for 1h. Then the immobilization of GOx onto coated and non-coated metal foams were determined. MG oxalate was used as a model for decolorization of biologic-catalytic combination process. The stock solution prepared in 80mg/L concentration, and the required solutions made from it. For each test, 25mL of the solution was transferred to the reactor to obtain decolorization, and the sampling was carried out at the specific times. Then, the variation of absorption at 617nm has measured as well the percentage of decolorization calculated by the following equation:

Conc.In specific time-Primary Conc.)/Primary Conc.= %decolor)

RESULT AND DISCUSSION

Characterization of Coated/uncoated Silver Metal Foam

FT-IR Spectroscopy was applied to confirm immobilization of GOx onto coated-SMF (Fig 2). The spectrum of the bare SiO_2 broad peak at 3435 corresponded to the stretching vibration of hydroxyl group while at 1635 may be attributed to the presence of stretching vibrations of Si-OH groups (Fig 2a) [37]. The band at 881 can be assigned to Si-O-Si symmetric stretching vibrations, whereas the IR band at 480 is due to O-Si-O bending vibrations. The very strong and broad band at 1059 comes from the symmetry vibration of the O-Si-O bond. The spectrum of silica-coated SMF (Fig 2b) shows changes compared to the structure of bare SiO_2 that are signs of the SMF cover. These are characterized by both the change of O-Si-O

bond main peak absorption at 1047 Moreover, the lower intensity of the peak at 3419.

After immobilization of GOx onto the SMF, the new absorption bands at 1095 and 2852 were observed (Fig 2c) [38]. The bands are related to the bending vibration and N-H band respectively, which are found in the enzyme structure and confirm the enzyme immobilization. The GOx-coated SMF shows an intense peak at 1647typical of amide (-C=O) band of the GOx and the broad peak at 3437 cm^{-1} was assigned the N-H/O-H stretching frequency of GOx. The CH_2 stretching of GOx moiety is related to the peak at 2852 cm^{-1}.

Fig. 3a-d shows SEM images in different scales of the (metal foam) MF before and after coating. As compared to the uncoated MF (Fig 3a-b), in the macrostructure of coated MF (Fig 3c), SiO_2 nanoparticles could be recognized on MF surface (Fig 3d). The efficiency of a sponge carrier was due to a high specific surface area, which improved attachment of the biomolecules and SiO_2 nanoparticles.

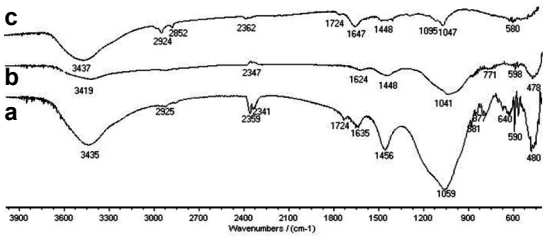

Fig. 2. FTIR spectra (a-c) for bare SiO_2, Silica-Coated SMF and GOx onto the SMF.

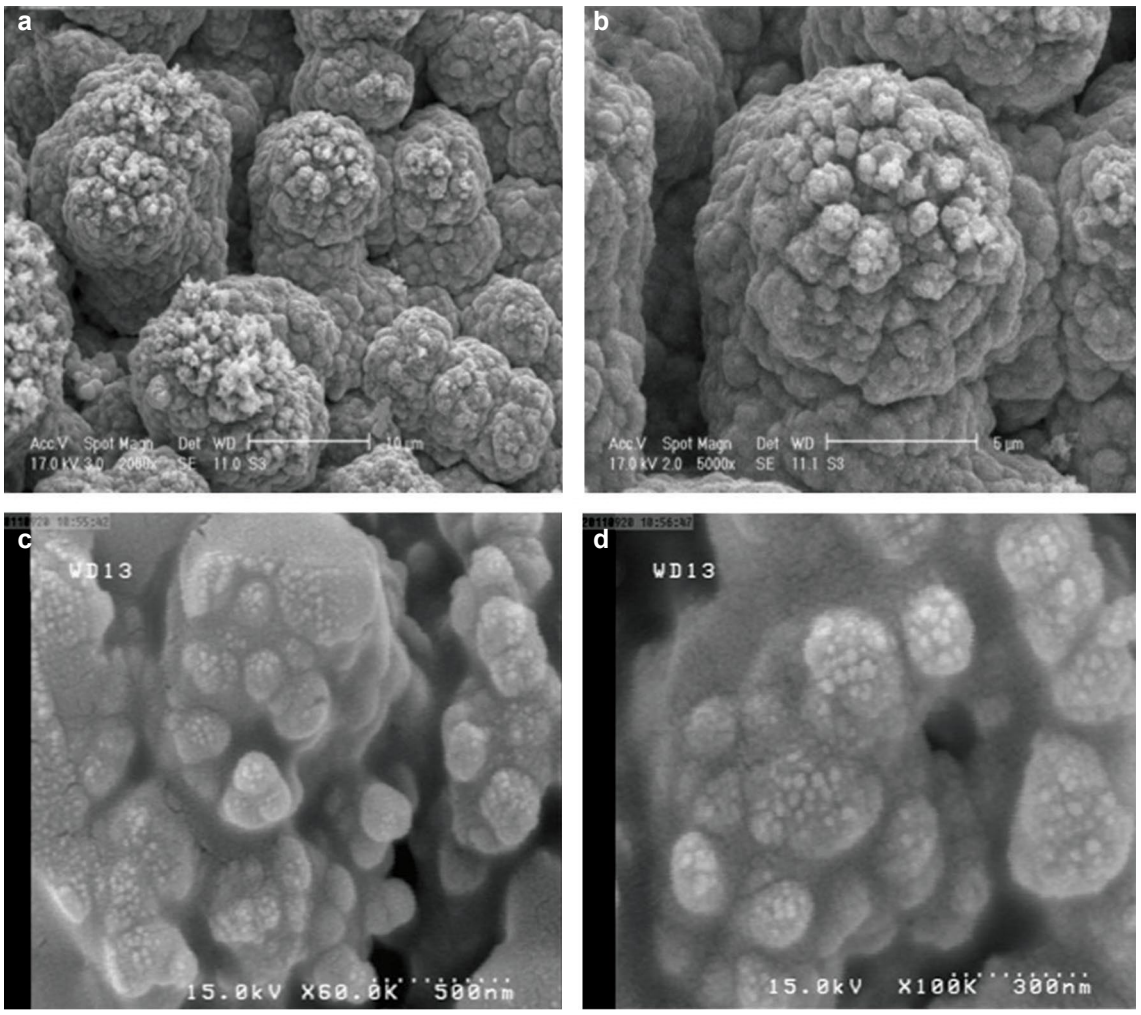

Fig. 3. Scanning electron microscopy (SEM) images of uncoated MF: (a-b) Structure of coated MF along with SiO_2 nanoparticles (c-d).

GOx activity onto coated/uncoated Silver Metal Foam

The physical adsorption method was employed in this work due to its simplicity and effectiveness of cost. The pH of the enzyme solution was adjusted to be the opposite charge between the enzyme and support.

Immobilized and free GOx activity was investigated at the same condition (i.e., pH=6.5 and T=40°C). As Fig.4 shows, 84% of the immobilized enzyme and 44% of the free enzyme activity have remained after 180 min. The free enzyme activity was about 61.5325 U/grSupport While the immobilized GOx activity was about 118.980 U/grSupport. The bonded enzyme onto the surface was obtained to be 0.09 mg/mL according to formula (1).

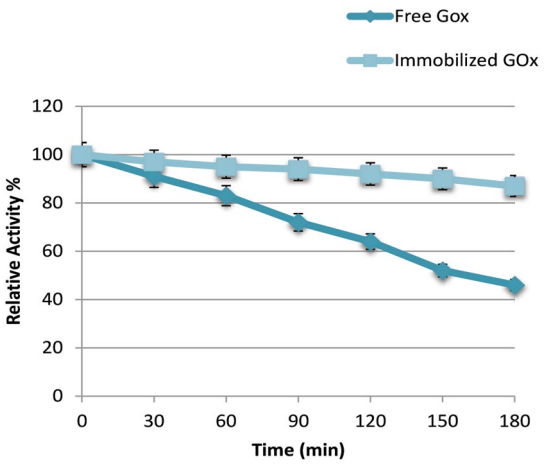

Fig. 4. Activity % of free and immobilized GOx at 40°C, pH=6.0 and different time.

Findings showed that immobilized GOx activity is two folds higher than free GOx after 180 min. The upper stability of immobilized GOx strongly depends on the support. Results are in good agreement with previous studies showed that immobilization of GOx on chitosan [39, 40], nickel-oxide (NiO) [41] and gold nanoparticles [42-44] lead to increasing the stability of the enzyme. A possible mechanism for increasing the stability of enzyme immobilization in porous support is capable of full dispersion of enzyme and also able to minimize possibly interacts with external interface [45, 46]. Furthermore, the immobilization of enzyme could avoid aggregation [47, 48] and decrease interacting with air bubble (i.e., H_2O_2 that produced by the catalytic activity of GOx) that effects on free enzyme activity [49-52].

Results showed the activity of immobilized GOx onto silica-coated SMF was increased near to 2 times more by the modification of the surface. Due to the accurate dimensions in nanometer scale, this type of foam is appropriate for stabilizing of enzymes while comparing to the coated foams. On the other hand, polymeric foams because of their lack of sufficient strength and ceramic foams, due to having no electrical properties of their surfaces cannot attach to the enzyme and should be used with coating materials as a medium. This may be for two main reasons: 1) The enzyme was effectively stabilized due to the high porosity of the silica structure [53, 54] 2) The bond strength of the surface was changed by silica-coated structure on the metal-foam surface. Moreover, the physical adsorption here ionic bond has been applied as the most successful and the cost effective method for enzyme activity improvement [55].

Decolorization

Elimination of MG depends on the hydrogen peroxide level produced by the immobilized enzyme. MG was prepared in four different concentrations 10, 20, 30 and 40 ppm to investigate the immobilized enzyme activity. The removal of MG was measured in 0, 15, 30, 45 and 60 min and dye absorbance was calculated by spectrophotometer (Fig. 5). The removal of MG was increased due to producing more hydrogen peroxide from enzyme by growing time. However, the increasing MG concentration results in dye elimination drop. Since enzyme concentrations were kept constant during this study, it is expected that increasing dye concentration did not affect decolorization level.

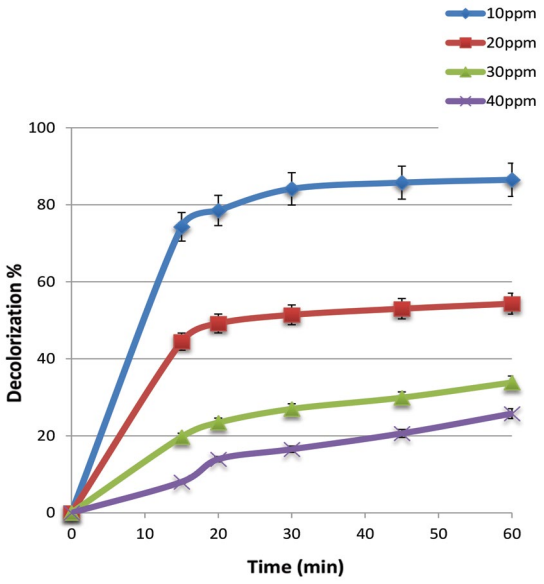

Fig. 5. Decolorization of different concentrations of MG at 40°C and pH=6.0.

Effect of temperature on GOx activity

Temperature is one of the significant factors in enzyme activity. The immobilized GOx illustrated more thermostability in comparison to the free one (Fig 6). It was previously discussed that the similar studies and possible mechanism according to these findings shown the molecular rigidity was occurred by the immobilized enzyme onto rigid support and formation of protected microenvironment [56-58].

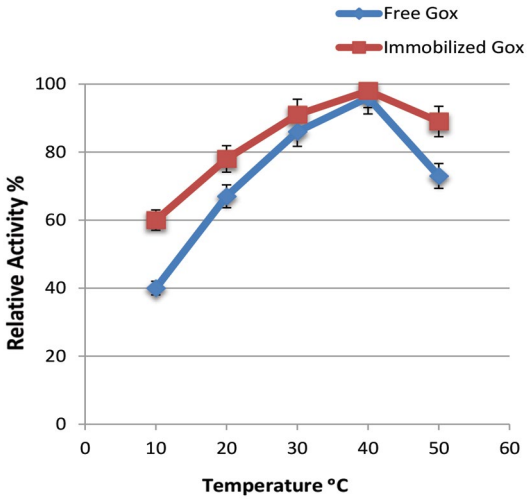

Fig. 6. Activity % of free and immobilized GOx at pH=6.0 and different temperature.

To confirm the effects of temperature on decolorization of MG, three experiments were performed at different temperatures 30°C, 40°C, and 50°C (Fig. 7). All temperature studies were carried out with 20ppm MG. The results revealed that the removal of MG increased at 40°C and decreased at 50°C by changing the temperature of MG solution. Also, the results obtained from fig.7 revealed that the highest removal of MG was achieved at 40°C. Since the optimum temperature of GOx was about 40°C, it is obvious that, with rising temperature, the rate of enzymatic reactions (Eq. (3)) Increased. In fact, the generation of hydrogen peroxide was elevated in the solution and would result in advanced elimination yield at any time during the process. Increasing temperature above optimum value could decrease the activity by changing the conformation of protein [59].

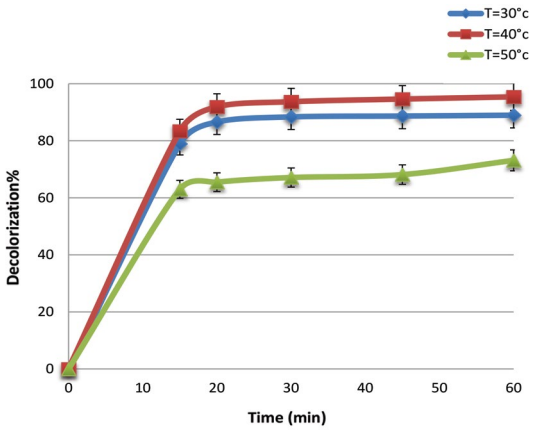

Fig. 7. Decolorization of MG at different temperature.

Reusability

Reusability plays a key role in the evaluation of immobilized enzyme while comparing to the free ones. The immobilized enzyme could remain onto the support by changing the reaction medium. In the present study, the reusability of immobilized GOx was carried out several times by using MG solution. After that, MG (20ppm) was added to GOx-SMF support at five stages and reusability of the immobilized enzyme was investigated by decolorization method. As detailed in fig .8, about 35% of enzyme activity remains after five stages shown that the immobilization of GOx onto SMF successfully improved the enzyme activity. According to the previous studies, the enzyme immobilization into rigid support leads to increase the interaction strength between support and enzyme [60, 61].

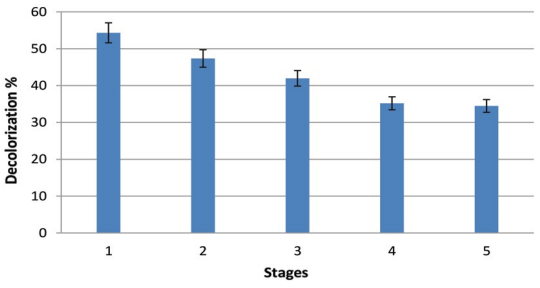

Fig. 8. Reusability of immobilized GOx onto SMF in 5 stages.

CONCLUSION

In this study, GOx was immobilized on SiO_2 coated and uncoated SMF to evaluate enzyme

activity. Then, obtained H_2O_2 was applied to an elimination process of MG. Findings showed that the immobilized GOx has more activity on SiO_2 coated SMF while compared to uncoated ones. The results obtained revealed that SiO_2 coated SMF could effectively be used to eliminate MG from solutions. Also, we believe that SMF can be applied as a suitable model for enzyme immobilization. However, more studies need to be performed to investigate the effects of other parameters and different coatings on immobilized enzyme activity onto SMF.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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