Composite Beads Containing Copper-metallothionein for the Catalase Immobilization

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Porous polysulfone (PSF) beads and composite beads (PSF-Cu-MT-Sil) obtained by incorporating copper-metallothionein functionalized silica in a polysulfone solution were prepared using the phase inversion method. Their characteristics were investigated with experiments of swelling, Fourier transform infrared (FTIR) spectroscopy and SEM microscopic analysis. Polysulfone and composite beads were used in catalase (CAT) immobilization. The kinetics of the biocatalytic process, stability under different temperature conditions and the inhibitors effect were studied as major factors on the immobilized catalase activity. The present work is meant to demonstrate the potential of crosslinked polysulfone and especially polysulfone with copper- metallothionein beads for the enzyme immobilization process.

Keywords: polysulfone, composite beads, catalase, enzyme immobilization

The development of the composite materials based on metallic complex has made a huge progress in the last years. These studies led to new synthesis procedures, capable of providing extended, stable wide pores structures, polymeric matrix and transitional metal ions in the polymeric matrix [1-5].

All these systems represent an important challenge for developing new types of composite materials. Several grafting procedures have been lately developed to covalently attach transition-metal complexes to organic polymers [6], silica, zeolites, and other micro- and mesoporous inorganic materials [7–9]. Copper (II) basis complex's (Schiff basis) immobilization on the porous supports, previously obtained [10] or prepared "in situ", in porous structure has been recently presented [11].

Among the polymeric compounds, chitosan is relative easily chelating with metallic salts due to the amino groups within its structure, forming polymer–complex metal type compounds [12], which are used as catalysts for organic synthesis, in medicine and in the drugs and enzymes delivery [13].

Polysulfone is one of the most commonly used polymers in preparing polymeric membranes due to its high mechanical and thermal stability and to its chemical resistance in acid and basic media, as well [14-16]. Recent studies revealed that composite membranes obtained from polysulfone solutions, including nanomaterials, have better properties than polymeric membranes [17]. One of the potential applications of the polysulfone membranes is the enzyme immobilization [18-20], but only few data can be found in literature concerning the polysulfone beads, or composite based on polysulfone beads preparation.

Catalase (EC 1.11.1.6), a heme containing metalloenzyme, is considered one of the most common enzymes in plant and animal tissues, having a protection function related to the decomposition of hydrogen peroxide.

With immobilized enzymes, improved stability, reusable capacity, continuous operation, possibility of reactions' better control, high purity and product yields, hence more favorable economic factors can be expected [21].

The preparation and characterization of the polysulfone (PSF) beads and of the composite beads (PSF-Cu-MT-Sil), obtained by incorporating copper-metallothionein functionalized silica in a polysulfone solution, were the main objective of our study. The other target was the catalase immobilization on these beads.

Experimental part

Preparation of silica functionalized with copper-metallothionein

Copper-metallothionein (Cu-MT) is characterised as a 53-residue polypeptide containing 12 cysteins and 8 copper atoms/molecule. Silica functionalized with Cu-MT was prepared as described in a previous publication [22].

The silica support was silanized with 3-aminopropyl-triethoxysilane (APTES) according to the previously described procedure [23]. Davicat Si-1452 silica, freshly activated overnight at 180°C under vacuum (1g), and APTES (1 mL) were mixed in 50 mL of dry toluene. After stirring the solution (reflux, 2 h), the released ethanol was distilled off and the mixture was kept under reflux for 90 min. The NH_2-functionalized silica (referred as Davicat-NH_2) was filtered and washed with toluene, ethanol and then diethyl ether. It was then submitted to a continuous extraction run overnight in a Soxhlet apparatus using diethyl ether/dichloromethane (v/v, 1/1) at 100°C and dried overnight at 130°C. The procedure of Cu-MT immobilization on the amino-functionalized support involves the mixing of 1g Davicat-NH_2 with 5 mL of 5% glutaraldehyde in 0.1 M sodium phosphate buffer (*p*H=7) for 30 min. Then, the excess of glutaraldehyde was removed during three cycles of centrifugation/washing with 10 mL buffer solution each. The Cu-MT in 14 mL 10mM Tris/HCl buffer (*p*H 7) was

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added to the activated solid. The suspension was stirred at 5°C for 24 h, centrifuged at 3000 rpm for 10 min to remove the buffer, and washed several times with buffer until no metallothioneine was detected in the washing. The solids thus obtained (referred as Cu-MT- Davicat-NH_2) were dried and stored at -5°C.

Preparation of polysulfone and composite beads

Polysulfone and composite beads have been obtained by phase inversion method [24, 25]. Polysulfone (PSF; M_n 22000) was dissolved in N,N-Dimethylformamid (DMF, Merck) to obtain the PSF solution 12% (w/v). Composite beads have been prepared by dispersion of Cu-MT silica (Cu-MT-Sil) into PSF solution. The polymeric solution was extruded dropwise through a syringe needle into the coagulation bath, and stirred about 300 rpm during 1 h to obtain the beads. In the first set of experiments coagulation was obtained in bi-distilled water and in the second, in a 1:1 (v/v) mixture of glutardialdehyde solution 2.5% and bi-distilled water.

The beads from the first experiment were incubated in cold 2.5% (w/v) glutardialdehyde solution for 1 h. Thereafter, the beads were washed with 100 mL 0.05M phosphate buffer solution, *p*H 7.0.

The diameters were 1.9± 0.2 and 2.6±0.3 mm for the beads prepared using polysulfone and polysulfone with Cu-MT-Sil, respectively.

The porosity of the microspheres were calculated from the density of the polymer and the weight change before and after drying, using the following formulas [26]:

$$Porosity,\% = \frac{(w_B - w_A)/\rho_w}{\frac{w_A}{\rho_p} + \frac{w_B - w_A}{\rho_w}} \cdot 100$$

where W_B is the weight of the beads before drying in grams; W_A is the weight of the beads after drying in grams; ρ_w is the density of water, ρ_w = 1.0 g/cm^3; ρ_{PSF} is the density of the polysulfone, ρ_{PSF} = 1.24 g/cm^3; and $\rho_{PSF\text{-}Cu}$ is the density of the polysulfone with Cu-MT-Sil, $\rho_{PSF\text{-}Cu}$ = 0.79 g/cm^3.

Structural characterization of polysulfone and composite beads

The morphology of beads was examined under scanning electron microscope using a ZEISS EVO LS10 microscope.

FTIR spectra were recorded on a FTIR spectrophotometer (Tensor 27 Brucker). The sample and KBr (1:5 ratio) were pressed to form a tablet.

Degree of swelling

The swelling rate of the porous polysulfone and composite beads in distilled water was determined by monitoring the weight gain of the porous beads in water, at 20°C for three days. The degree of swelling (S_w) was calculated according to:

$$S_w = \frac{(W - W_0) \cdot 100}{W_0}$$

where W and W_o denote the weight of porous beads with absorbed water and that of the dry porous beads, respectively.

Catalase immobilization on polysulfone and composite beads

Immobilization experiments were conducted for 24 h at 20°C. Polysulfone and composite beads (50 mg PSF and PSF-Cu-MT-Sil) were immersed in 1 mL of catalase solution (0.5 mg/mL) in 0.05 M phosphate buffer *p*H 7.0. After the immobilization period, polysulfone and composite beads (PSF-CAT and PSF-Cu-MT-Sil -CAT) were removed from each solution and washed several times with 0.05 M (3 mL) phosphate buffer solution (*p*H 7.0). The immobilized preparations were stored at 4°C in the phosphate buffer (*p*H = 7). Total concentration of catalase in the solutions was determined by the Lowry method using a UV-VIS Jasco spectrophotometer [27].

The immobilization yield was defined as follows:

$$\text{Immobilization yield } \% = \frac{AE_{imm}}{AE_{free}} \times 100$$

where AE_{imm} is the total activity of immobilized enzyme (U/mg) and AE_{free} is the total activity of the initial enzyme preparation (U/mg).

Enzyme activity assay

Hydrogen peroxide solutions (5-20 mM) were used to determine the catalytic activity. Catalase free (0.1mL), catalase immobilized on PSF and PSF-Cu-MT-Sil beads (50 mg) were mixed with 2.9 mL of hydrogen peroxide solution in 50 mM phosphate buffer (pH 7.0) at 25°C and a decrease in absorbance at 240 nm for 3 min was recorded [28, 29]. The free and immobilized catalase activities were calculated.

Kinetic parameters of the enzymatic reactions

The Michaelis–Menten kinetic parameters K_M and v_{max} of the free and immobilized enzymes were determined by varying hydrogen peroxide concentrations from 5 to 20 mM at 25°C, in phosphate buffer *p*H 7. The kinetic parameters were transformed to Lineweaver–Burk plots and values were calculated from the slopes and intercepts of the curves.

Thermal and storage stabilities

The thermal stabilities of the free and immobilized catalase were determined by measuring the residual enzymatic activity (with hydrogen peroxide substrate) at different temperatures (20, 30, 40, 50 and 60°C) in phosphate buffer (50 mM, *p*H 7.0), incubated for 60 min. For testing the enzymes storage stability, free and immobilized catalase in 50 mM phosphate buffers (*p*H 7.0) were stored for twenty days in a refrigerator at 4°C. Then the activity of the enzyme was measured at 25°C in standard conditions. Each set of experiments was carried out in triplicate, the mean values and standard deviations were calculated and the limits of error range for each data set were determined using the statistical package under Excel (Windows).

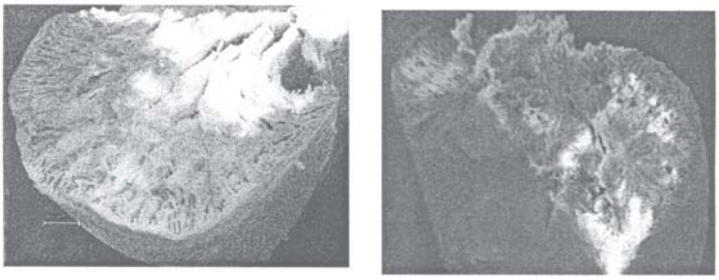
Inhibition of catalase

In the present paper we studied the effect of some organic compounds (acetylsalicylic acid and 2-chloroethylamine hydrochloride) on catalase activity. Catalase free and catalase immobilized were incubated for 2 h, with 1 mL inhibitor compound (2-chloroethylamine hydrochloride 0.5% w/v and acetylsalicylic acid 1% w/v). The reaction ran at 25°C, started by the addition of the substrate (hydrogen peroxide solution in 50 mM phosphate buffer) and the result is the absorbance at 240 nm decrease recording. A control test, when the inhibitor compound was

Sample	Mean size, mm	Porosity, %
PSF_1	1.9±0.2	85.2±0.3
PSF_2	2.0±0.2	81.7±0.1
PSF-Cu-MT-Sil_1	2.6±0.3	82.1±0.2
PSF-Cu-MT-Sil_2	2.7±0.3	79.3±0.1

Data are expressed as the means ± S.D. of independent measurements. (n=5)

Table 1
DIAMETER AND POROSITY OF THE POLYSULFONE AND COMPOSITE BEADS



a) PSF bead b) PSF-Cu-MT-Sil bead

Fig. 1 SEM images of PSF and composite beads

replaced by buffer, was conducted in parallel. The inhibition percentage was calculated by means of the formula:

$$I\ \% = \frac{A_{inh}}{A_0} \cdot 100$$

where I= inhibition (%), A_{inh} = absorbance in inhibition test after 3 min, and $A_{0\,=}$ absorbance in control test after 3 min.

Results and discussion

The polysulfone and composite beads

In this study we prepared 4 types of beads: PSF beads coagulated in bi-distilled water (PSF_1) and in glutardialdehyde solution (PSF_2), composite beads coagulated in bi-distilled water (PSF-Cu_MT-Sil_1) and in glutardialdehyde solution (PSF-Cu_MT-Sil_2), respectively. The beads prepared using 12% PSF solution were used in all experiments. The diameter and porosity of the prepared beads are shown in table 1.

The influence of the added glutardialdehyde in the coagulation solution can be observed by means of beads' porosity decrease, as compared to those obtained when the coagulation was performed in bidistilate water.

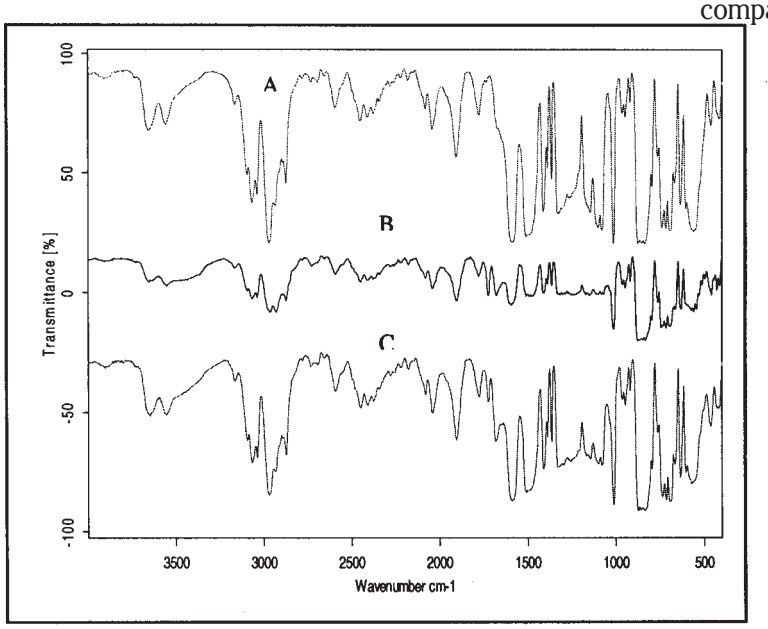
Figure 1 present SEM micrographs of the morphology for the porous polysulfone and for the composite beads cross sections. A skin layer was found on the outer surface of the bead, and many pores in the bead.

The FTIR spectra (fig. 2 a and b) showed adsorption bands assigned to the polysulfone. The 1506/1508 and 1592 cm^{-1} bands are due to the aromatic hydrocarbons, the 2968cm^{-1} bands are assigned to the aliphatic hydrocarbons and the 1320/1364 cm^{-1} band are assigned to the diaryl sulphones. However, from 1800 to 4000 cm^{-1}, the spectra showed different band intensities but with little differences among them.

Figure 2b shows that the PSF-Cu-MT-Sil have new peaks appearing at 1608 cm^{-1}, at 1721 cm^{-1} and a peak at 555 cm^{-1}, almost arising from the metallothionein.

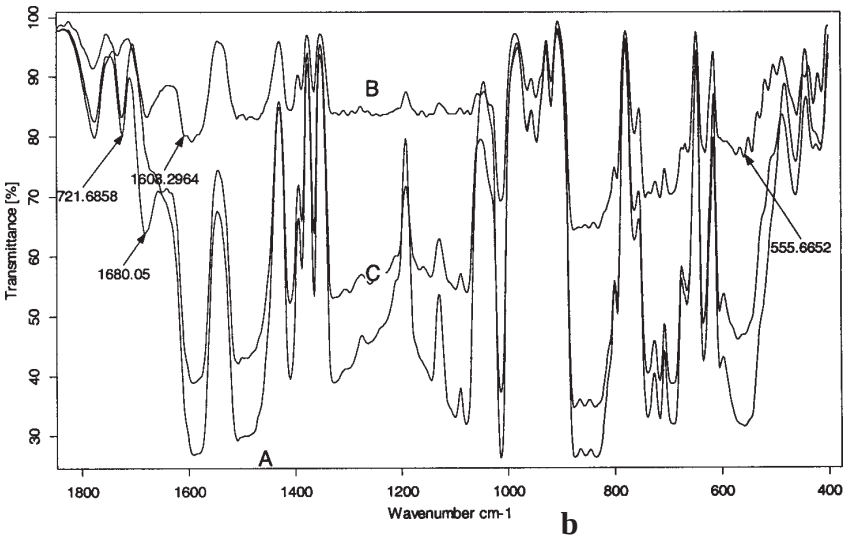
Catalase from bovine liver has several histidin and tryptophan residues and has a characteristic absorbtion band at 1678 cm^{-1} representing amide bond. The peaks between 3450 and 3500cm^{-1} were due to N–H stretch. It was shown that the intensity of peak at 1680 cm^{-1} increased in the presence of copper and catalase and proved the bands between protein and support. This increase indicates the protein binding to the matrix.

The swelling curves of the beads in distilled water are shown in figure 3. As one can see in the figure, the beads swell, approach the equilibrium-swelling value in distilled water after about 24 h and then reached a constant value. The swelling degree for PSF beads was very small as comparing to the PSF-Cu-MT-Sil beads.



a

Fig. 2a. FTIR spectra of PSF(A), PSF-Cu-MT-Sil (B), PSF-Cu-MT-Sil –CAT (C)



b

Fig. 2b. FTIR spectra of PSF(A), PSF-Cu-MT-Sil (B), PSF-Cu-MT-Sil –CAT (C)

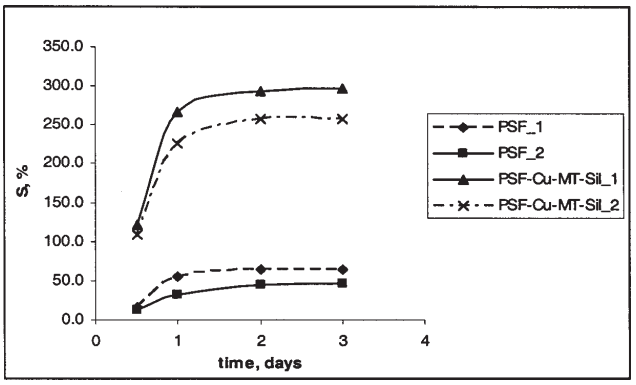


Fig. 3. Swelling curves of the PSF and PSF-Cu-MT-Sil beads

Catalase Immobilization

The enzyme was immobilized by means of adsorption on a simple solid support or on a solid support cross-linked with glutardialdehyde, when polysulfone beads are used. In case of composite beads (PSF-Cu-MT-Sil), catalase can interact from various sites. Firstly, ε-amino group of Lys residues on catalase surfaces can bind free aldehyde groups of glutardialdehyde (free amino groups of metallothionein form Schiff's bases with glutardialdehyde). Accordingly, imidazolyl, thiol and indolyl groups of His, Cys, Trp (respectively) residues on protein surfaces can coordinate with Cu from the beads.

Catalase immobilization experiments were performed at *p*H 7.0 in 50 mM phosphate buffer.

Immobilization yields were calculated as 74.2±2.0 % and 93.2±1.0% for PSF and PSF-Cu-MT-Sil, respectively.

Kinetic parameters of the enzymatic reactions

Kinetic of free and immobilized catalase catalytic activities was investigated at various concentrations (5–20 mM) of hydrogen peroxide (in 50 mM phosphate buffer at *p*H 7.0), as a substrate. These data were plotted according to Lineweaver–Burk and kinetic parameters, K_M (for free enzyme), apparent K_M (for immobilized enzyme) and v_{max}, were calculated from the graphs (table 2).

As expected, the K_M and v_{max} values were significantly affected after immobilization on PSF and PSF-Cu-MT-Sil beads. The increase in apparent K_m and decrease in v_{max} values for immobilized catalase suggested that the immobilized catalase on beads had a lower affinity for binding substrate. The change in the affinity of the enzyme to its substrate is probably determined by structural changes in the enzyme, introduced by the immobilization procedure or by lower accessibility of the substrate to the active site of the immobilized enzyme. In this study, we found that the v_{max} value of PSF-Cu-MT-Sil-CAT was higher than that of the PSF-CAT, because the amount of immobilized protein of PSF-Cu-MT-Sil -CAT was higher than that of PSF-CAT.

A similar result involving changes in K_M and v_{max} values of enzyme after immobilization has been reported in literature [30-32].

Thermal and storage stabilities

Figure 4 illustrates the effect of temperature on the immobilized catalase activity. The optimum temperature was 30°C for free catalase and PSF-CAT and 25–30°C for PSF-Cu-MT-Sil-CAT. Residual activities for free catalase, immobilized catalase on PSF beads and immobilized catalase on PSF-Cu-MT-Sil beads were 91, 93 and 95% of their original activity at 30°C, after 60 min. preincubation time.

PSF-Cu-MT-Sil-CAT showed a high stability at 25–30°C. Free catalase and PSF-CAT showed high stability at 30°C.

Table 2
PROPERTIES OF THE FREE AND IMMOBILIZED CATALASE

	K_M^*, (mM)	v_{max}, U/mg protein	Bound protein, mg/g carrier
Free catalase	43.4±0.6	125±3.8 ×10^3	-
PSF_1 - CAT	55.5±0.9	16±0.2 ×10^3	0.249±0.02
PSF_2 - CAT	53.8±0.9	18±0.3 ×10^3	0.254±0.01
PSF-Cu-MT-Sil_1 – CAT	91.0±1.8	30±0.5 ×10^3	0.415±0.03
PSF-Cu-MT-Sil_2 – CAT	90.4±1.6	33±0.7 ×10^3	0.428±0.03

Data are expressed as the means±S.D. of independent measurements (n=3).
*K_M for free enzyme and K_M apparent for immobilized enzyme.

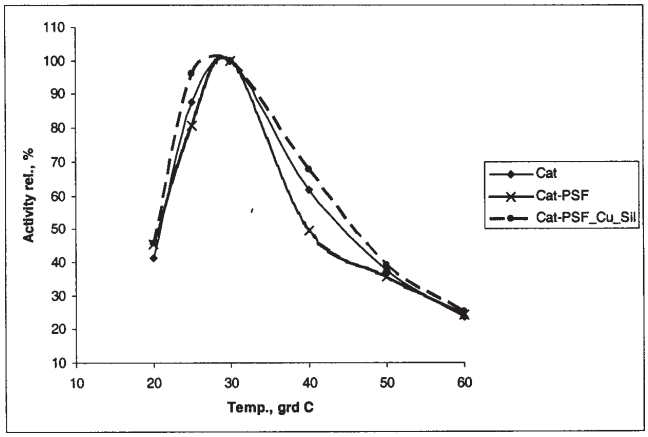


Fig. 4. Effect of temperature on the catalytic activities of catalase

Table 3
INHIBITION EFFECT OF DIFFERENT COMPOUNDS ON FREE AND IMMOBILIZED CATALASE

	I (%)	
	2-chloroethylamine hydrochloride	acetylsalicylic acid
Free catalase	59.7	85.9
PSF-CAT	59.9	76.5
PSF-Cu-MT-Sil-CAT	75.2	68.8

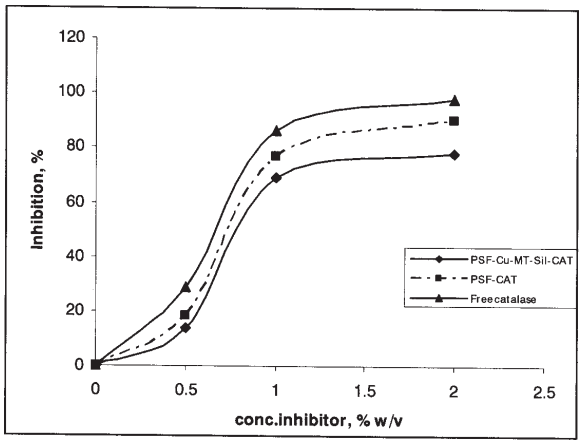


Fig.5. Effect of acetylsalicylic acid on soluble and immobilized catalase

Generally, when incubation time increased the catalytic activities of free and immobilized enzyme decreased. This situation was more evident at higher temperatures (35–60°C).

The storage stabilities of PSF-CAT and PSF-Cu-MT-Sil-CAT were approximately the same. At 4°C, free enzyme lost about 50% of its initial activity within 20 days, whereas PSF-CAT and PSF-Cu-MT-Sil-CAT stored within 20 days under similar temperature conditions lost only 20% of their initial activity. Also the immobilized catalase showed better thermal and storage stabilities.

Inhibition of catalase

The action of 2-chloroethylamine hydrochloride 0.5% w/v and acetylsalicylic acid 1% w/v, as possible inhibitors of the free and immobilized catalase activity, was studied. Our results showed that 2-chloroethylamine hydrochloride has a slight inhibition activity, while acetylsalicylic acid has a strong inhibition activity on the catalase (table 3).

The effect of various concentrations of acetylsalicylic acid (0.5–2.0%, w/v) on the activity of catalase was also studied (fig. 5). The incubation of free catalase with 2.0% acetylsalicylic acid for 2 h at 25°C resulted in a significant loss of 98% of the initial activity, while catalase immobilized on PSF and PSF-Cu-MT-Sil beads lost 90% and 78% of the initial activity, under similar exposure.

Our results are in agreement with those reported in literature [33, 34].

Conclusions

A new type of composite beads, obtained by incorporating copper-metallothionein functionalized silica in a polysulfone solution, was successfully prepared using the phase inversion method. Catalase was successfully immobilized on the polysulfone and composite beads, using quite simple method. Immobilized protein amount and reaction maximum velocity for PSF-Cu-MT-Sil-CAT were higher than for PSF-CAT. In both situations, catalytic activities of the immobilized catalase were lower than that of free catalase, but PSF-Cu-MT-Sil-CAT showed a high temperature stability and storage stability. Cross-linked polysulfone and especially polysulfone with copper-metallothionein beads have demonstrated a good potential to be used for enzyme immobilization.

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