Journal of Molecular Catalysis B: Enzymatic 60 (2009) 69–75

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Optimum immobilization of urease on modified acrylonitrile copolymer membranes: Inactivation by heavy metal ions

Katya Gabrovska, Tzonka Godjevargova *

University "Prof.Dr.A.Zlatarov", Department of Biotechnology, Prof Y. Yakimov Str. 1, 8010 Bourgas, Bulgaria

ARTICLE INFO

Article history:
Received 1 October 2008
Received in revised form 25 March 2009
Accepted 27 March 2009
Available online 5 April 2009

Keywords:
Membrane
Chemical modification
Immobilization
Urease
Optimization
Heavy metal ions

ABSTRACT

Poly(acrylonitrile-methylmethacrylate-sodium vinylsulfonate) membranes were subjected to chemical modification with hydroxyl ammonium sulfate ($NH_2OH{\cdot}H_2SO_4$) and the amount of functional amidoxime groups was determined in the modified membranes. Urease was covalently immobilized on the modified membranes. The relationship between immobilization factors and enzyme activity was examined by a series of contour plots. The selections of the immobilization variable range were extremely precise in the 3-level-3-factor fractional design. The results indicated that the optimal conditions for urease immobilization were: 0.1% enzyme solution, immobilization temperature – 4 °C and immobilization time – 20 h.

The inhibitory effect (IC_{50}) of Cu(II), Cd(II), Zn(II), Ni(II) and Pb(II) was studied on free and immobilized urease. The behavior of the immobilized urease in model solutions, containing different mixtures of heavy metals was represented in a 3D model. By studying the inhibition effect of two different mixtures Cu(II) and Cd(II); Cu(II) and Zn(II) it was found that the linear range of urease inhibition by Cu(II) ions for the first model mixture was from 0 to 1 mg l^{-1} and by Cd(II) ions from 0 to 1.5 mg l^{-1}, as for the second model mixture the linear range of urease inhibition by Cu(II) ions was from 0 to 1 mg l^{-1} and by Zn(II) ions from 0 to 3.0 mg l^{-1}. This linear dependence is very important from a practical point of view, regarding the application of immobilized urease in the construction of a biosensor for the detection of low concentrations of heavy metal ions.

1. Introduction

Enzymes, which are usually used as biocatalysts in biochemical processes, are preferred to chemical catalysts because they are more selective and more efficient. Immobilization is usually considered to be an important technique to enhance the stability of enzymes. Actually, enzyme stability is greatly dependent on the immobilization strategy [1–3].

Obviously, the support used in enzyme immobilization is important since its interaction with enzyme may have an influence on the stability and reaction kinetics [4–6]. As support matrices, polymeric membranes have attracted much attention because they can be produced easily in a wide variety of compositions and can be modified for immobilization of biomolecules by introducing a wide variety of ligand molecules [7–10]. Due to their biocompatibility acrylate polymers are regarded as suitable matrices for enzyme immobilization. Among them, poly(acrylonitrile) polymers are very versatile and convenient due to their hydrophilic nature, good blood compatibility, high chemical and mechanical stability and resistance toward microbial and enzymatic attacks [11–13]. Non-modified membranes of acrylonitrile (AN) copolymer lack suitable active groups for enzyme immobilization. Considerably active nitrile groups, present in AN copolymers, allow additional functional groups to be introduced by chemical polymer reactions [14,15]. The chemically modified membranes of AN copolymer have good chemical and mechanical stability and are not susceptible to microbial attack.

A few publications deal with covalent immobilization of enzymes onto chemically modified membranes of AN copolymer [16]. Among the different immobilization techniques, covalent attachment may have a higher commercial potential than other methods because it is simpler and less expensive and a high catalytic activity may be retained. The method also ensures the reusability of the expensive supports after the inactivation of the immobilized enzyme [17–19].

For further industrial applications, it is important to design an efficient enzymatic immobilization system and to obtain optimum immobilization conditions in a short period of time with minimum trails. It is very important to know the conditions under which the greatest specific enzyme activity and activity yield will be achieved. These conditions are specific for each carrier and enzyme [3].

* Corresponding author. Tel.: +359 56 858353; fax: +359 56 880249.
E-mail address: godjevargova@yahoo.com (T. Godjevargova).

1381-1177/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2009.03.018

Urease (EC 3.5.1.5; urea amidohydrolase) is a nickel-dependent enzyme [20] that catalyzes the hydrolysis of urea to form ammonia and carbon dioxide. Membrane-immobilized urease opened the way for constructing urea sensors, heavy metal sensors and membrane bioreactors applied for urea determinations and urea removal [21,22], respectively. In all processes mentioned above the enzyme is exposed to inhibition by different heavy metal ions [23]. The inhibition of urease provides a sensitive screening test for copper and mercury [24,25] and, with lower sensitivity, for nickel, cadmium, lead and zinc [26]. Activity reduction of free or immobilized urease in the presence of different concentrations of heavy metal ions may be used for quantitative analyses of heavy metal solutions [27].

In the present study, urease was covalently immobilized on a modified AN copolymer membrane. One of the objectives of this work was to better understand the relationships between the immobilization variables (immobilization time, immobilization temperature, and enzyme concentration) and the response (enzyme activity) and to obtain the optimum conditions for urease immobilization. The other objective consisted in studying the inhibition potency of several heavy metal ions (single and combined) and assessing the adequacy of the obtained enzyme system for the preparation of urease biosensor for the determination of low concentrations of heavy metal ions.

2. Materials and methods

2.1. Chemicals

Surface modifications of AN copolymer (PAN) membranes were carried out using the following agents: hydroxyl ammonium sulfate ($NH_2OH{\cdot}H_2SO_4$), produced by Fluka, Switzerland.

Immobilization of jack bean urease (EC 3.5.1.5, 270 U/mg, 480,000 Da, Merck, Germany) onto the PAN membranes was carried out with glutaraldehyde (Fluka, Switzerland).

The following salts were used for the inactivation of free and immobilized urease: $CuSO_4{\cdot}5H_2O$, $CdCl_2{\cdot}2{,}5H_2O$, $Ni(NO_3)_2{\cdot}6H_2O$, $Zn(NO_3){\cdot}6H_2O$, $Pb(NO_3)_2$. All the salts were from Fluka, Switzerland.

All other reagents were of analytical grade.

2.2. Membranes

Poly(acrylonitrile-methylmethacrylate-sodium vinylsulfonate) membranes used in the experiments, were provided by Spartak, Bulgaria. The ternary copolymer (acrylonitrile – 91.3%; methylmethacrylate – 7.3%, sodium vinylsulfonate – 1.4%) was a product of Lukoil Neftochim, Bourgas.

Ultrafiltration membrane of acrylonitrile copolymer was used, which could retain substances with molecular weight higher than 10,000 Da. The specific area of the membrane was 10 $m^2\,g^{-1}$ and the mean pore size was 0.02 μm.

2.3. Chemical modifications of PAN membranes

Chemical modification of PAN membrane was carried out in the presence $NH_2OH{\cdot}H_2SO_4$. The optimum conditions for the membrane chemical modification were determined in our previous works [16,31].

The amounts of functional amidoxime groups in the modified membranes were measured by residual titration in heterogeneous medium [28]. A piece of membrane (1 cm^2) was placed in 10 ml 0.025 M solution of HCl with added 0.005 M NaCl. The sample was stirred for 24 h on a magnetic stirrer in order to completely neutralize the amidoxime groups on the membrane surface. The excess of HCl was titrated with a 0.05 M solution of NaOH in the presence of an indicator methylrot.

2.4. Immobilization of urease on PAN membrane

The immobilization of urease on PAN membrane was realized with 12% solution of glutaraldehyde and urease solutions with varying concentrations in sodium phosphate buffer with pH 5.8 [31]. The optimum conditions for urease immobilization were determinated by incrementing the immobilization time from 15 to 25 h, immobilization temperature from 0 to 8 °C and enzyme concentration from 0.05 to 0.15%. The relationship between immobilization factors and urease activity was assessed by using the statistical analysis and was presented in three-dimensional graphs.

2.5. Activity assay

The membrane with the immobilized enzyme was immersed in a 0.1 M solution of urea in 0.06 M sodium phosphate buffer, pH 7.0, 30 °C (0.1 M urea in 0.06 M sodium phosphate buffer, pH 5.8, 28 °C for free urease). After 5 min of incubation the amount of released NH_3 was determined spectrophotometrically at wavelength 460 nm (Specol 11, Carl Zeiss Jena, Germany) by measuring the intensity of the coloured compound formed after the addition of Nessler's reactant.

The relative activity was determined as the ratio between the specific activity of a bound enzyme and the specific activity of the free enzyme, multiplied by 100.

2.6. Statistical analysis

A 3-level-3-factor fractional design was employed in this study, requiring 14 experiments. The experimental data were analyzed by the response surface regression (RSREG) procedure to fit second-order polynomial equation (SAS) [29].

2.7. Protein determination

The amount of protein bound to the modified membranes was determined by the method of Lowry. The method is based on spectrophotometric measurements of the blue colour resulting from the interaction of cupric ions with peptide bonds in alkali medium and from the reaction of the aminoacidous residues with Folin reactant [30].

2.8. Inhibition by heavy metal ions

The inhibition effect of Cu(II), Cd(II), Ni(II), Zn(II) and Pb(II) ions over the activity of the immobilized urease was determined through estimation of the activity reduction in the presence of different concentrations of these metal ions [27]. The immobilized urease was incubated in a predefined concentration of a given metal ion in bidistilled water, pH 7.0 for 30 min. For free urease the pH of heavy metal solutions (prepared with bidistilled water) was adjusted with a HCl solution to pH 5.8.

Enzyme inhibition was determinated as follows:

$$[\%] = \left(\frac{a_o - a_i}{a_o}\right) \times 100$$

where a_o – activity without inhibition and a_i, – enzyme activity after incubation with samples.

To train and test the neural networks, inhibition effect on immobilized urease by two different mixtures was studied. The first binary mixture consisted of Cu(II) in the concentration range 0.1–2.0 mg l^{-1} and Cd(II) in the concentration range 0.1–3.0 mg l^{-1}. The second binary mixture consisted of Cu(II) and Zn(II) in the same concentration range.

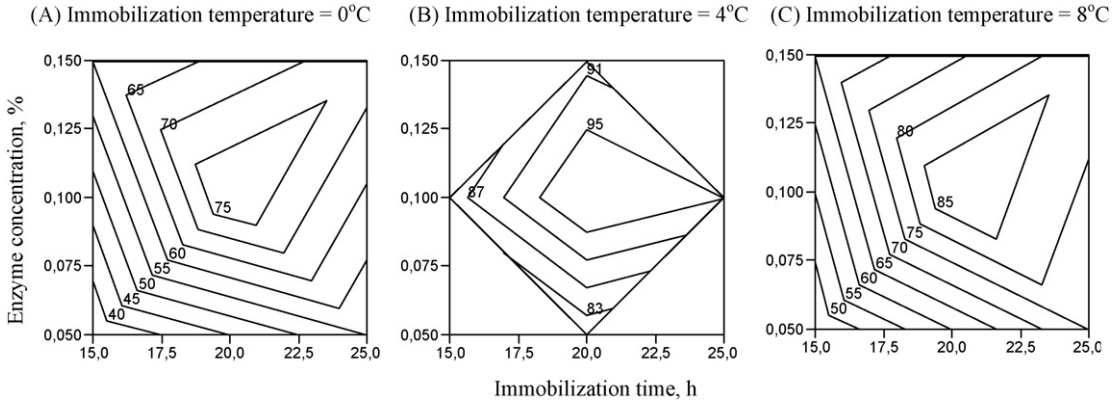

Fig. 1. Contour plots showing response behavior at varying immobilized times and enzyme concentrations under constant immobilized temperature for urease-PAN membrane. (A) Immobilization temperature = 0 °C, (B) 4 °C, and (C) 8 °C.

3. Results and discussion

3.1. The optimal condition for urease immobilization

The PAN membrane was modified by hydroxyl ammonium sulfate [31]. This modification was selected to obtain amidoxime groups, which can covalently bind urease, by crosslinking with glutaraldehyde. The amount of functional amidoxime groups in the modified membranes were determined—0.18 mgequiv g^{-1}.

The proposed method involves the following reactions (Eqs. (2) and (3)):

$$\text{PAN–NH}_2 + \text{OHC(CH}_2)_3\text{CHO} \underset{-\text{H}_2\text{O}}{\longrightarrow} \text{PAN–N=CH–(CH}_2)_3\text{–CHO} \quad (2)$$

$$\text{PAN–N=CH–(CH}_2)_3\text{–CHO} + \text{H}_2\text{N–}E \underset{-\text{H}_2\text{O}}{\longrightarrow} \text{PAN–N=CH–(CH}_2)_3\text{–CH=N–}E \quad (3)$$

where E stands for enzyme.

For industrial application, it is important to design an efficient enzyme system and to obtain the optimum immobilization conditions in a brief period of time with minimum effort. The main objectives of this work were to better understand relationships between the immobilization factors and the relative activity of enzyme in order to establish the optimum conditions for urease immobilization.

3.1.1. Model fitting

The relationship between immobilization factors and response can be better understood by examining the series of contour plots. The selection of the immobilization variable range needs to be extremely precise in the 3-level-3-factor fractional design. The RSREG procedure for Statistical Analysis System (SAS) was employed in order to fit a second-order polynomial Eq. (4) to the experimental data, represented as activity (% of maximum). From the SAS output of RSREG, the second-order polynomial Eq. (4) is given below:

$$\begin{aligned} Y = {} & 48.605 + 65.579x_1 - 1.011x_2 - 7.689x_3 + 78.125x_1x_2 \\ & - 99.375x_1x_3 - 421.875x_2x_3 - 46.875x_1x_2x_3 \\ & - 3.755x_1^2 - 8.993x_2^2 - 32.375x_3^2 \end{aligned} \quad (4)$$

The analysis of variance indicate that the model was statistically significant and adequate to represent the actual relationship between the response (relative activity) and significant variables, with fairly small p-value (0.0027) and small coefficient of determination (R^2 = 0.99).

3.1.2. Mutual effect of parameters

The variables and their levels selected in this study were: immobilization time (15–25 h), immobilization temperature (0–8 °C), enzyme concentration (0.05–0.15%). The effect of varying immobilization time and enzyme concentration is shown in Fig. 1. At any given time from 15 to 20 h, an increase of enzyme concentration led to a higher activity (% of maximum). A condition with immobilization time 20 h and enzyme concentration of 0.1% favored maximum activity (over 80% at 0 and 8 °C and 100% at 4 °C). Fig. 2 represents the effect of varying immobilization temperature and enzyme concentration. A condition with temperature of 4 °C and

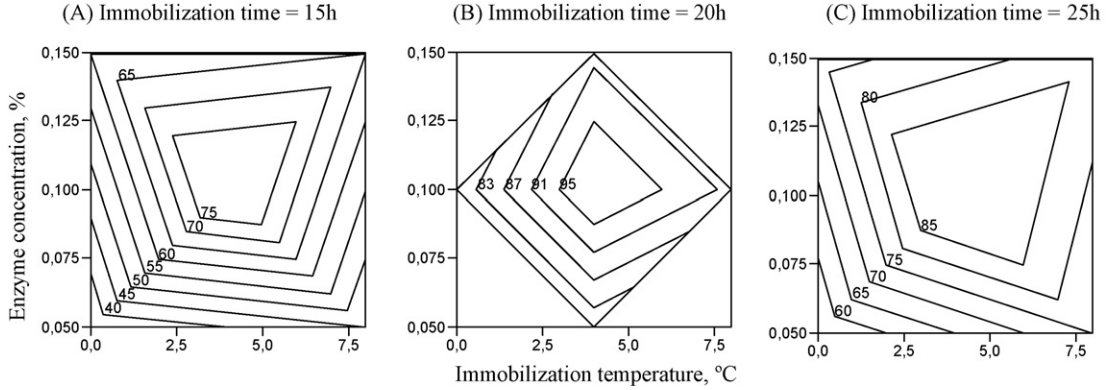

Fig. 2. Contour plots showing response behavior at varying immobilized temperatures and enzyme concentrations under constant immobilized time for urease-PAN membrane. (A) Immobilization time = 15 h, (B) 20 h, and (C) 25 h.

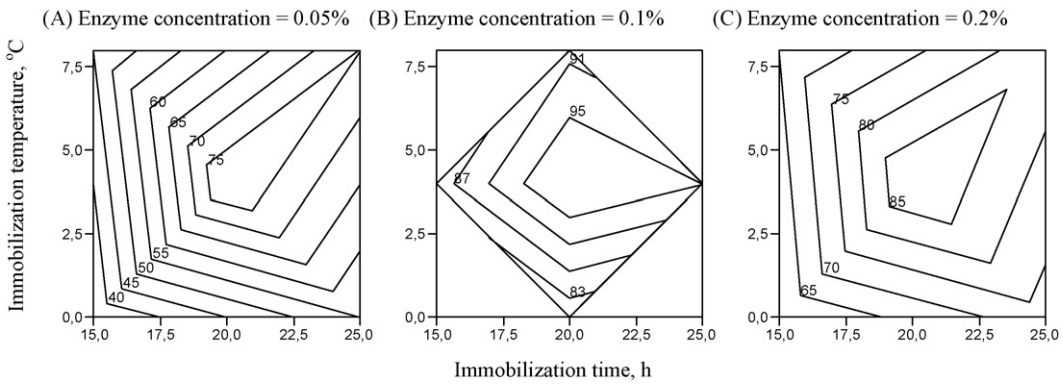

Fig. 3. Contour plots showing response behavior at varying immobilized times and temperatures under constant enzyme concentration for urease-PAN membrane. (A) Enzyme concentration = 0.05%, (B) 0.1%, and (C) 0.2%.

enzyme concentration of 0.1% led to maximum activity (over 75% at 15 and 25 h and 100% at 20 h). Fig. 3 shows the effect of immobilization time, temperature and their mutual interaction on urease activity. A condition with immobilization time of 20 h and temperature of 4 °C led to the maximum activity (over 75% at enzyme concentration – 0.05 and 0.15% and 100% at enzyme concentration – 0.1%).

Such an application could be adopted to study the immobilization variables simultaneously in a three-dimensional space. Immobilization time and enzyme concentration were the most important variables for the immobilization conditions with small *p*-values and considered as indicators of effectiveness.

The analysis indicated that the maximum activity of 100% was achieved at 20 h, 4 °C and enzyme concentration 0.1%. Under those conditions the relative activity of the immobilized urease was 80%, and the bound protein – 0.029 mg cm^{-2}.

3.2. The inhibition effect of heavy metals on immobilized and free urease

It has been known that heavy metal ions are inhibitors of urease activity, and it has been suggested that this inhibition may involve blockage of thiol groups in the protein. However, some metal ions do not react only with thiol groups, but also with N- and O-containing ligands [32,33]. The heavy metal ions such as Cu(II), Zn(II), Ni(II), Pb(II) belong to the borderline group of acids in the Hard and Soft Acids and Basics (HSAB) theory. They exhibit a high affinity to N- and O-containing ligands, though they are capable of formation of stable complexes with all groups of ligands depending on availability. The inhibition of urease activity by heavy metal ions has been proposed as non-competitive type [31], partial competitive [34] and mixed [35]. More recently, it has been established that this inhibition appears to be more complex and it has also been shown that it is slow-binding [36]. On the basis of activity reduction of free and immobilized urease in the presence of different concentrations of heavy metal ions some analytical methods can be developed pointed at quantitative determination of heavy metal ions. The reusability of the immobilized urease, makes it preferable for application in continuous processes.

For that reason, the inhibitory effect of Cu(II), Cd(II), Zn(II), Ni(II) and Pb(II) on free and immobilized urease was studied (Fig. 4). The free urease assay showed that it was most sensitive to copper and least to lead. According to their inhibition effect over free urease the heavy metal ions could be ordered in the following sequence:

$$Cu(II) > Cd(II) > Zn(II) > Ni(II) > Pb(II).$$

Fig. 4 shows that the inhibition activity of the investigated metals had lesser effect toward the urease membrane, modified with $NH_2OH{\cdot}H_2SO_4$. This protection may result from the structural changes introduced to the enzyme structure by the applied immobilization procedure and consequently providing lower accessibility to the essential –SH groups of the enzyme active site [37]. According to their inhibition effect on the immobilized enzyme the heavy metal ions could be ordered in the same sequence as the case with free urease. Fahmy et al. [38] investigated the inhibitory effect of some heavy metal ions at the concentration of 1×10^{-3} M on *C. vulgaris* urease. They arranged these ions in order of decreasing toxicity:

$$Hg(II) \sim Ag(I) > Cu(II) > Zn(II) > Ni(II) > Cd(II) > Co(II) > Fe(III) > Mn(II)$$

with 100, 100, 94.3, 86.7, 85.0, 82.7, 80.0, 69.5 and 44% inhibition, respectively. Regarding the immobilized enzyme, the inhibitory effect of these metal ions at the same concentration was in the order:

$$Hg(II) > Ag(I) > Cd(II) > Co(II) > Ni(II) > Cu(II) > Zn(II) > Mn(II) > Fe(III)$$

with 98.0, 86.2, 75.0, 70.0, 68.0, 65.0, 63.0, 33.0 and 22.0% inhibition, respectively. The improved stability of the immobilized urease turned the enzyme to be more resistant to the inactivation by the heavy metal ions. Krajewska [37] inactivated the immobilized jack bean urease on glutaraldehyde-pretreated chitosan membrane with heavy metal ions and compared the results obtained for the free enzyme. The relative toxicity sequence of metal ions toward free urease was found to be

$$Hg(II) > Ag(I) > Cu(II) > Ni(II) > Cd(II) > Zn(II) > Co(II) > Fe(III) > Pb(II) > Mn(II).$$

They found that the stability of jack bean urease against metal ion inactivation was considerably improved after immobilization, whereas Ni(II), Co(II), Fe(III), Pb(II) and Mn(II) did not inactivated the immobilized urease in the range of concentrations from 1 to 5×10^{-5} M, at which free urease was thoroughly inactivated. Hg(II), Ag(II), Cu(II), Zn(II) and Cd(II) inactivated the immobilized enzyme at considerably low concentrations (1×10^{-7} M).

In this study the concentration of heavy metal ions which giving 50% inhibition of enzyme activity was determinated (IC_{50}) (Table 1). The free urease assay was most sensitive to copper (IC_{50} = 0.018 mg l^{-1}) and least to lead (IC_{50} = 9.92 mg l^{-1}). IC_{50} of the other metals were in the interval from 0.5 to 1 mg l^{-1}. The obtained values of IC_{50} for each metal were compared to analogous results, published by other authors (Table 1). As can be seen, the free urease, used in our experiments, has shown less sensitivity to the inhibition activity of the heavy metals than the urease, used by Jung et al.

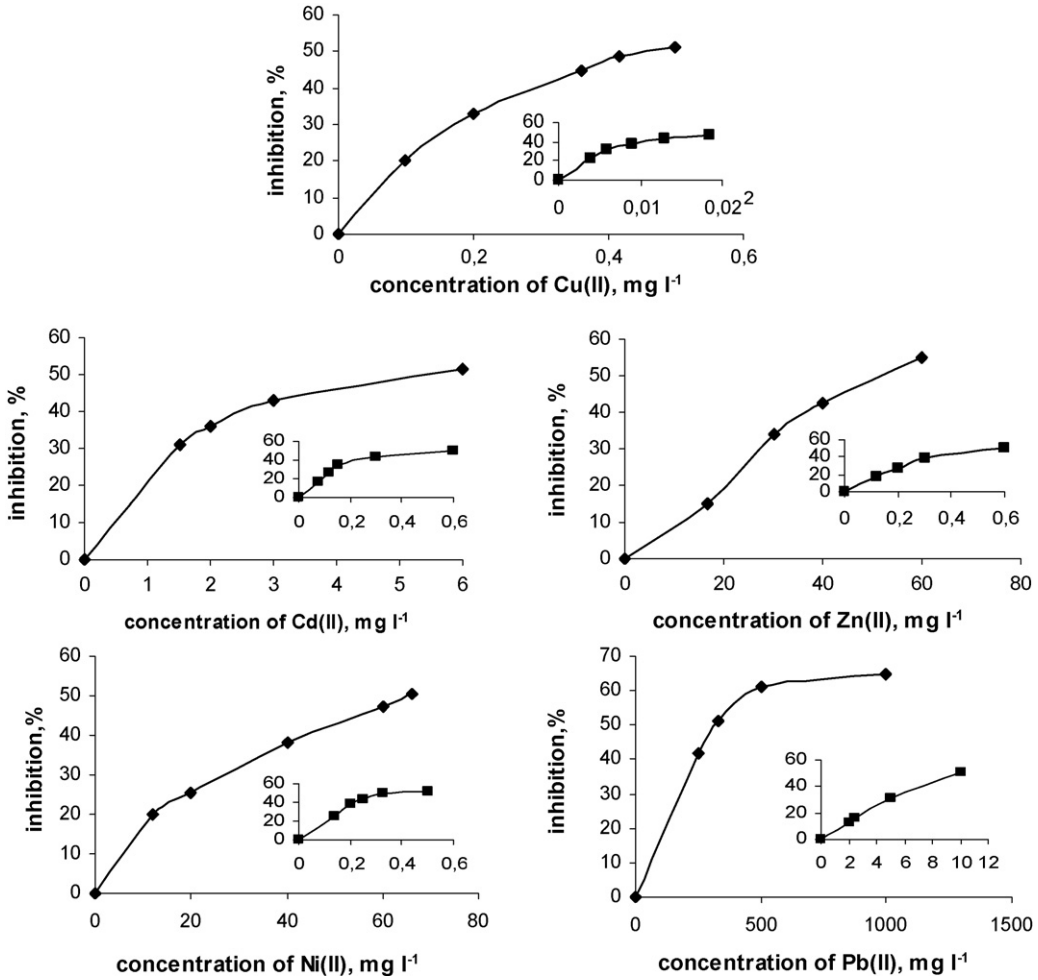

Fig. 4. Inhibition of free (■) and immobilized (♦) urease on PAN membrane modified with $NH_2OH{\cdot}H_2SO_4$ by heavy metal ions.

[27] (Table 1). According to other authors [39–41], urease has been less inhibited by cupric ions (IC_{50} = 0.06 mg l^{-1}), and the rest of the values of IC_{50} for the other metal ions with regard to the free urease have been greater than the results obtained during the present study by 1–2 orders of magnitude.

In our case it was found that IC_{50} for the urease immobilized on modified PAN membrane was greater (1–2 orders of magnitude) than IC_{50} of the same ions for the free enzyme (Table 1). A higher stability of the immobilized urease toward the inhibition activity of heavy metals has also been reported by Jung et al. (Table 1) [27]. It is noticeable that some IC_{50} values, reported by those authors, are considerably lower with regard to Ni(II), Pb(II) and Zn(II) than the results from the present work for immobilized urease. When comparing the experimental values of IC_{50} with analogous results, reported by other authors [38] a prominent difference has been observed only for Cd(II) and Cu(II), whereas the results concerning Ni(II) and Zn(II) have appeared to be similar. Such difference has not been observed for the free urease. A plausible reason could be the type of the carriers and their charge. AN copolymer membrane modified with hydroxyl ammonium sulfate, contains positively charged amidoxime groups. Cu(II), Ni(II), Pb(II) and Zn(II) metal ions readily connect to the rest of amidoxime groups after the enzyme immobilization and form strong chelate complex, which is the reason for the higher values of IC_{50} regarding the interaction between the immobilized urease and those metals (Table 1). For that reason the urease, immobilized on the AN copolymer membrane, modified with hydroxyl ammonium sulfate, showed greater stability toward the heavy metal inhibitors. Krajewska [37] has also reported a lesser selectivity of chitosan immobilized urease toward heavy metals, unlike free urease, which is attributed, by the authors, to the chitosan chelate-bonding incline.

From a practical point of view it is very important to study the behavior of the immobilized urease on membranes modified with $NH_2OH{\cdot}H_2SO_4$ in model solutions, containing different mixtures of heavy metals. The first series of experiments were based on model solutions of Cu(II) ions with concentrations ranging from 0.1 to 2.0 mg l^{-1} and Cd(II) ions with concentrations ranging from 0.1 to 3 mg l^{-1}. The second series of experiments investigated the model solutions of Cu(II) and Zn(II) ions within the same concentration intervals. The metal ions and their concentrations were chosen so that to represent real wastewater samples from the galvanic industry. Furthermore these two metals, Cd(II) and Zn(II) are among the strongest urease inhibitors, as can be seen from the inhibition activity sequence, mentioned above. That is why their complemen-

Table 1
IC_{50} comparison of heavy metal ions of free and immobilized urease.

Heavy metal ions	IC_{50} (mg l^{-1})			
	Free urease	Free urease [27]	Immobilized urease	Immobilized urease [27]
Cu(II)	0.018	0.013	0.49	0.41
Cd(II)	0.59	0.12	5.85	1.59
Zn(II)	0.6	0.18	53.1	14.6
Ni(II)	0.91	0.51	65.2	1.52
Pb(II)	9.92	2.5	409	>250

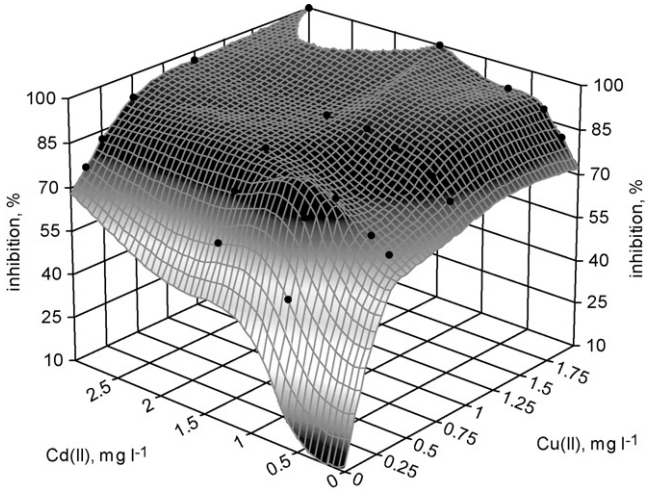

Fig. 5. 3D plot showing urease inhibition of binary mixture consisting of Cu(II) and Cd(II) ions.

tary influence over the strong inhibition activity of Cu(II) would be the most prominent. The results of the two experiment series are presented in Figs. 5 and 6. As can be seen the overall inhibition was determined by the mutual activity of the two metals from the mixture, i.e. the overall inhibition effect is the total value of the inhibition activities of each metal ion.

Figs. 5 and 6 clearly show that the inhibition activity of Cd(II) was greater than Zn(II). The maximum inhibition due to the combined activity of Cu(II) and Cd(II) toward the immobilized urease was 90% (Fig. 5), whereas the overall inhibition of the model solution of Cu(II) and Zn(II) reached the maximum of 70% (Fig. 6). Since the inhibition activity of Zn(II) was not significant in the concentration range 0.1–3.0 mg l^{-1}, the main inhibition activity toward immobilized urease was contributed to Cu(II) ions. As mentioned above Cu(II) are the strongest urease inhibitor among the investigated ions and they constitute the major part of the overall inhibition effect of the investigated solution, containing Cu(II) and Zn(II). Because of the complementary activity of Zn(II), the registered inhibition effect of the model mixtures slightly surpassed the inhibition effect of the solution, containing only Cu(II).

Figs. 5 and 6 bring information about the interval of linear dependence between metal ion concentration and the degree of immobilized urease inhibition. This linear dependence is very

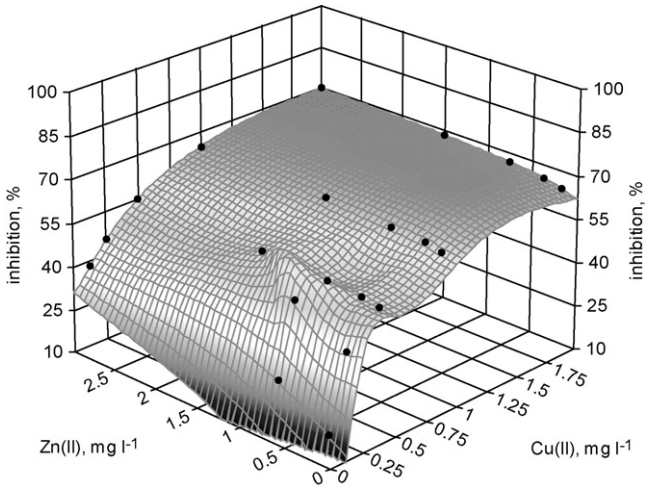

Fig. 6. 3D plot showing urease inhibition of binary mixture consisting of Cu(II) and Zn(II) ions.

important from a practical point of view, regarding the application of immobilized urease in the construction of a biosensor for the detection of low concentrations of heavy metal ions. As can be seen from Fig. 5 the linear range of urease inhibition by Cu(II) ions was from 0 to 1 mg l^{-1} and by Cd(II) ions from 0 to 1.5 mg l^{-1}. As for the second model mixture the linear range of urease inhibition by Cu(II) ions was from 0 to 1 mg l^{-1} and by Zn(II) ions was from 0 to 3.0 mg l^{-1} (Fig. 6). These initial results show that the obtained immobilized system has a good potential and after additional researches can be applied for the construction of a biosensor for a quantitative determination of heavy metal ions.

4. Conclusion

The optimum conditions for urease immobilization on modified PAN membrane were determinated and suggested by the ridge max analysis: 0.1% enzyme solution, immobilization temperature – 4 °C and immobilization time – 20 h. At those conditions the based characteristics of immobilized urease are: relative activity – 80%, bound protein – 0.029 mg cm^{-2}.

By studying the inhibition effect of two different mixtures Cu(II) and Cd(II); Cu(II) and Zn(II) it was found that for the first model mixture the linear range of urease inhibition by Cu(II) ions was from 0 to 1 mg l^{-1} and by Cd(II) ions was from 0 to 1.5 mg l^{-1}, as for the second model mixture the linear range of urease inhibition by Cu(II) ions was from 0 to 1 mg l^{-1} and by Zn(II) ions was from 0 to 3.0 mg l^{-1}. This linear dependence is very important from a practical point of view, regarding the application of immobilized urease in the construction of a biosensor for the detection of low concentrations of heavy metal ions.

References

[1] R.A. Sheldom, Adv. Synth. Catal. 349 (2007) 1289–1307.
[2] L.Q. Cao, Curr. Opin. Chem. Biol. 9 (2005) 217–226.
[3] W. Tischer, F. Wedekind, Top. Curr. Chem. 200 (1999) 95–126.
[4] J.M. González-Sáiz, C. Pizarro, Eur. Polym. J. 37 (2001) 435–444.
[5] E. Agostinelli, F. Belli, G. Tempera, A. Mura, G. Floris, L. Toniolo, A. Vavasori, S. Fabris, F. Momo, R. Stevanato, J. Biotechnol. 127 (2007) 670–678.
[6] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Enzyme Microb. Technol. 40 (2007) 1451–1463.
[7] H.T. Deng, Z.K. Xu, J. Wu, P. Ye, Z.M. Liu, P. Seta, J. Mol. Catal. B: Enzym. 8 (2004) 95–100.
[8] K. Gabrovska, T. Nedelcheva, T. Godjevargova, O. Stoilova, N. Manolova, I. Rashkov, J. Mol. Catal. B: Enzym. 55 (2008) 169–176.
[9] E. Magnan, I. Catarino, D. Paolucci-Jeanjean, L. Preziosi-Belloy, M.P. Belleville, J. Membr. Sci. 241 (2004) 161–166.
[10] Z.M. Liu, S. Tingry, C. Innocent, J. Durand, Z.K. Xu, P. Seta, Enzyme Microb. Technol. 39 (2006) 868–876.
[11] H.G. Hicke, P. Böhme, M. Becker, H. Schulze, M. Ulbricht, J. Appl. Polym. Sci. 60 (1996) 1147–1161.
[12] M. Ulbricht, A. Papra, Enzyme Microb. Technol. 20 (1997) 61–68.
[13] T. Godjevargova, R. Dayal, I. Marinov, J. Appl. Polym. Sci. 91 (2004) 4057–4063.
[14] W. Chaing, C. Hu, J. Appl. Polym. Sci. 28 (1990) 1623–1626.
[15] K. Matsumoto, R. Izumi (1983) US patent 4,486,549.
[16] T. Godjevargova, K. Gabrovska, J. Biotechnol. 103 (2003) 107–111.
[17] G. Bayramoglu, M.Y. Arica, Biochem. Eng. J. 20 (2004) 73–77.
[18] B. Rehbock, R.G. Berger, Biotechnol. Tech. 12 (1998) 539–544.
[19] N. Fatma, F. Bozoglu, V. Hasirci, Biosens. Bioelectron. 17 (2002) 531–539.
[20] N.E. Dixon, C. Gazzola, R.L. Blakeley, B. Zerner, J. Am. Chem. Soc. 97 (1975) 4131–4133.
[21] T.C. Huang, D.H. Chen, Biotechnol. Bioeng. 40 (1992) 1203–1209.
[22] S. Akgol, Y. Yalcinkaya, G. Bayramoglu, A. Denizli, M.Y. Arica, Process Biochem. 38 (2002) 675–683.
[23] B. Rodriguez, J. Bolbot, I. Tothill, Biosens. Bioelectron. 19 (2004) 1157–1167.
[24] A. Soldatkin, V. Volotovsky, A. El'skaya, N. Jaffrezic-Renault, C. Martelet, Anal. Chim. Acta 403 (2000) 25–29.
[25] B. Krajewska, W. Zaborska, M. Chudy, J. Inorg. Biochem. 98 (2004) 1160–1168.
[26] W. Brack, A. Paschke, H. Segner, R. Wennrich, G. Schüürmann, Chemosphere 40 (2000) 829–834.
[27] K. Jung, G. Bitton, B. Koopman, Water Res. 29 (1995) 1929–1933.
[28] K. Dimov, V. Sarmadjieva, P. Pavlov, Laboratory Practices on Technology of Synthetic Fibres, Technica, Sofia, 1983, pp. 145–146.
[29] SAS/SAS User Guide, SAS Institute Inc., Cary, North Carolina, 1990.
[30] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.

[31] T. Godjevargova, K. Gabrovska, Macromol. Biosci. 5 (2005) 459–466.
[32] B. Krajewska, J. Enzyme Inhib. Med. Chem. 23 (2008) 535–542.
[33] C. Follmer, C.R. Carlini, Arch. Biochem. Biophys. 435 (2005) 15–20.
[34] K. Oehlschläger, R. Hüttl, G. Wolf, Thermochim. Acta 310 (1998) 185–189.
[35] M.L. Antonelli, V. Carunchio, M. Luciani, G. Vinci, Thermochim. Acta 122 (1987) 95–103.
[36] W. Zaborska, B. Krajewska, Z. Olech, J. Enzyme Inhib. Med. Chem. 19 (2004) 65–69.
[37] B. Krajewska, J. Chem. Tech. Biotechnol. 52 (1991) 157–162.
[38] A. Fahmy, V. Bagos, T. Mohammed, Bioresour. Technol. 64 (1998) 121–129.
[39] D.L. Olson, G.M. Christensen, Bull. Environ. Contam. Toxicol. 28 (1982) 439.
[40] W.H.R. Shaw, J. Am. Chem. Soc. 76 (1954) 2160–2163.
[41] U. Blum, G. Schwedt, Acta Hydrochim. Hydrobiol. 26 (1998) 235–239.