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OPTIMIZATION OF ENZYME IMMOBILIZATION ON INTERPOLYMER MEMBRANES

The paper presents some preliminary results of assessing the efficiency of papain immobilization on amine modified polyethylene/poly(styrene-co-divinylbenzene) and polyethylene/poly(vinylbenzyl chloride-co-divinylbenzene) membranes. The enzyme was immobilized due to adsorption. Three process variables were analyzed: concentration of papain in solution, time and temperature of sorption. In order to find the optimal values of these variables, statistical procedure was applied. It was shown that temperature insignificantly affected immobilization and its role could be accepted. The values of concentration and the time that allowed obtaining the most active preparations were also established.

Keywords: *interpolymer membrane, immobilization, papain, optimization*

1. INTRODUCTION

To reduce both capital and recurrent costs of enzyme-catalyzed processes some enzymes are often used in their immobilized forms. The main purpose of immobilization is to obtain industrially suitable biocatalyst, i.e. such preparation that can be reused in large-scale processes. Immobilization enhances also enzyme stability and prevents the final product from contamination. Among a great number of support matrices used for this task, polymers have been applied extensively for the last decades. The reason for so great interest can be explained by the fact that these materials are not very expensive, but are available in large qualitates and can bear a lot of various functional groups [1]–[3].

We applied weak-basic anion-exchange membranes from interpolymer of polyethylene/poly(styrene-co-divinylbenzene) (PE/poly(S-co-DVB)) and polyethylene/poly-

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(vinylbenzyl chloride-co-divinylbenzene) (PE/poly(VBC-co-DVB)) as the supports for immobilization of papain (EC 3.4.22.2). Papain is proteolytic enzyme which catalyzes the breakdown of proteins into short-chain peptides, amino acid esters and amides. For this reason, it is widely used in food industry and medicine [4].

The optimization of immobilization process may significantly reduce its cost. In order to develop an efficient strategy of immobilization of papain on weak-basic membranes, modelling of response surface may be developed. This statistical method was already used for optimization of the growth and enzyme production by a fungus *Fusarium colorum* [5]. The procedure should allow identifying the factors that are responsible for biomembrane activity.

2. EXPERIMENTAL

2.1. MATERIALS

N-*α*-benzoil-*L*-arginine ethyl ester hydrochloride (BAEE) was manufactured by Sigma, Germany; other reagents were supplied by POCh, Poland. Papain [EC 3.4. 22.2] was from Fluka AG, Switzerland.

2.2. MEMBRANES

Styrene or vinylbenzyl chloride and divinylbenzene were polymerized in low-density polyethylene according to method described earlier [6]. The content of DVB in the monomer mixture was equal to 2 wt.-% and the concentration of poly(S-co-DVB) or poly(VBC-co-DVB) in the interpolymers was set at 30 wt.-%. The PE/poly(S-co-DVB) interpolymer was chloromethylated with methyl chloromethyl ether in the presence of $SnCl_4$ as a catalyst under conditions established elsewhere [7]. A weak-basic membrane from the PE/poly(S-co-DVB) interpolymer was obtained via amination of the chloromethylated intermediate with 1,2-diaminoethane [8] – membrane A and from the poly(VBC-co-DVB) interpolymer via direct amination – membrane B.

The chloromethylation of aromatic polymers has been a key step in the production of anion exchangers and membranes. Methyl chloromethyl ether has been very successfully applied in the chloromethylation reactions, providing excellent conversion and high yields. However, taking into account the carcinogenicity of this reagent, some alternative methods of chloromethylation have been employed lately [9]. One of them is the use of vinylbenzyl chloride (interpolymer B) in place of styrene (interpolymer A). It was found that the membrane B showed comparable ion exchange capacity – 0.80 mmol/g dry mem-

brane, whereas this capacity of the membrane A was 0.62 mmol/g dry membrane.

2.3. IMMOBILIZATION

Papain was immobilized due to adsorption. Each membrane of the area of 10 cm^2 was immersed in 5 cm^3 of aqueous solution of papain. The concentration of the enzyme, contact time and temperature were altered as required for a design protocol. After immobilization the membranes were rinsed several times with distilled water.

2.4. ANALYTICAL METHODS

The amount of immobilized papain was determined by mass balance before and after the immobilization process. For this purpose protein assay test was applied according to Lowry's method [10]. The efficiency of immobilization was determined as the esterolytic activity of immobilized enzyme, using BAEE as a substrate. The concentration of the remaining substrate was determined colorimetrically according to Brown's method [11]. One unit of enzyme activity was defined as the activity causing the hydrolysis of 1 μmol of BAEE during 1 minute.

2.5. OPTIMIZATION PROCEDURE

STATISTICA, Release 4.0 (StatSoft Inc.) software was used for designing an experimental protocol, analysis of the data obtained and calculation of the surface of response. The design of protocol was selected to show the statistical significance of the process variables, i.e., papain concentration, contact time and temperature, to the activity of immobilized enzyme. This allows us to estimate the polynomial relationships between the factors evaluated and the dependent variable and gives additionally an insight into correlation of independent factors.

3. RESULTS AND DISCUSSION

In our investigation of the performance of the biofunctional membrane, it has been hypothesized that there are three major immobilization factors affecting activity of the immobilized enzyme: enzyme concentration, contact time and temperature. The ranges of their variances were evaluated previously. The summary of an experimental design is given in tables 1 and 2 for membranes A and B, respectively.

The experiments were carried out in random order, and the STATISTICA program generated the levels of independent factors in a single experiment.

Table 1

Immobilization of papain on the membrane A. Results of experimental design

No.	Concentration of papain [mg /cm^3]	Immobilization time [h]	Temperature [^{o}C]	Activity [U/m^2]
1	6.5	5.2	24	130.0
2	6.5	8.8	24	137.5
3	19.5	5.2	24	120.0
4	19.5	8.8	24	140.0
5	6.5	5.2	36	132.5
6	6.5	8.8	36	137.5
7	19.5	5.2	36	112.5
8	19.5	8.8	36	140.0
9	13.0	7.0	30	180.0
10	13.0	7.0	30	175.0
11	13.0	10.0	30	102,5
12	13.0	7.0	30	130.0
13	2.0	7.0	30	135.0
14	24.0	7.0	30	87.5
15	13.0	7.0	20	180.0
16	13.0	7.0	40	177.5
17	13.0	7.0	30	182.5

Table 2

Immobilization of papain on the membrane B. Results of experimental design

No.	Concentration of papain [mg/cm^3]	Immobilization time [h]	Temperature [^{o}C]	Activity [U/m^2]
1	3.2	6.5	24	125.0
2	6.8	6.5	24	117.5
3	3.2	19.5	24	95.0
4	6.8	19.5	24	160.0
5	3.2	6.5	36	125.0
6	6.8	6.5	36	105.0
7	3.2	19.5	36	95.0
8	6.8	19.5	36	160.0
9	5.0	13.0	30	175.0
10	5.0	13.0	30	175.0
11	2.0	13.0	30	90.0
12	8.0	13.0	30	142.5
13	5.0	2.0	30	82.5
14	5.0	24.0	30	95.0

15	5.0	13.0	20	207.5
16	5.0	13.0	40	160.0
17	5.0	13.0	30	175.0

In order to estimate the effect of the factors under investigation on the preparation activity, the ANOVA analysis of variance has been applied (see data in tables 3 and 4).

Table 3

Analysis of variance. Papain immobilized on the membrane A

Effect	Sums of squares $SS\times10^{-4}$	Mean square error $MS\times10^{-4}$	p level
Concentration	1.32	1.32	0.015
Time	1.29	1.29	0.016
Temperature	0.01	0.01	0.779
Square of concentration	9.20	9.20	0.000
Square of time	10.70	10.70	0.000
Square of temperature	0.00	0.00	0.852
Concentration – time	0.25	0.25	0.196
Concentration – temperature	0.01	0.01	0.842
Time – temperature	0.02	0.02	0.692
Residuals	0.69	0.12	

The statistical significance of the relations estimated is given by p level which represents the probability of error that allows accepting our results as valid, that is, as representative of the whole population. We set the p level at 0.05 as a boundary value for an acceptable error.

The results obtained for membrane A (table 3) indicate that two factors play a crucial role in enzyme immobilization: concentration of papain (significance level $p = 0.015$) and contact time (significance level $p = 0.016$). Both of them affect activity also in square relations (significance level $p = 0.00$). None of the rest bilateral interactions has influenced the activity of immobilized enzyme.

Table 4

Analysis of variance. Papain immobilized on the membrane B

Effect	Sums of squares $SS\times10^{-4}$	Mean square error $MS\times10^{-4}$	p level
Concentration	4.26	4.26	0.002
Time	4.10	4.10	0.044
Temperature	1.00	1.00	0.158
Square of concentration	8.48	8.48	0.000
Square of time	17.78	17.78	0.000
Square of temperature	0.09	0.09	0.483

Concentration – time	4.96	4.96	0.001
Concentration – temperature	0.03	0.03	0.669
Time – temperature	0.03	0.03	0.669
Residuals	0.93	0.15	

Table 4 shows the results of ANOVA calculated for the membrane B. In this case, the enzyme concentration and time were also found to be the most important factors. The squares of concentration and time affect the enzyme activity ($p = 0.00$) and their bilateral interaction ($p = 0.01$).

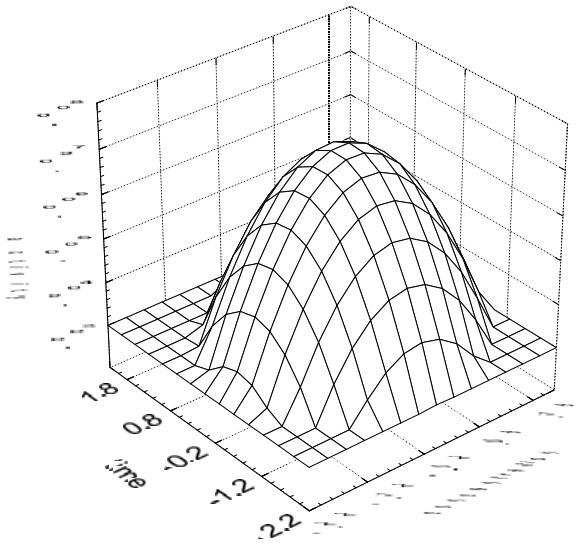

Fig. 1. Effect of papain concentration and contact time on the activity of immobilized enzyme

The surface plot calculated from data of table 1 and governed by two variables, i.e. concentration and time, is shown in figure 1. The vertical axis shows the expected activity of immobilized papain. A detailed presentation of the optimum being predicted based on the experimental data is given in figure 2.

The maximum activity of the immobilized enzyme–membrane preparation (membrane A) corresponds to the point representing the enzyme concentration of 7.3 mg/cm^3 and the time of 12.5 hours. The same procedure was applied in order to evaluate the factors of the membrane B. The concentration calculated and the time were found to be 5.8 mg/cm^3 and 14.3 hours, respectively.

In the case of both of the membranes tested, an increase in the papain concentration and the sorption time made the biofunctional membrane more active. However, after exceeding the maximum, a further increase in the papain activity was not obtained. The same phenomenon was observed for trypsin immobilized on polymer ma-

trices [12]. The authors of that paper have explained it by such an accumulation of protein molecules on the polymer surface that reduces the substrate accessibility. It was also observed that the amidase activity of papain is much higher at low enzyme load when homogeneous or immobilized preparations were applied [13]. Papain, the proteolytic enzyme, may self-digest or forms inactive dimers. Both of these processes result in a decrease in the activity observed [12].

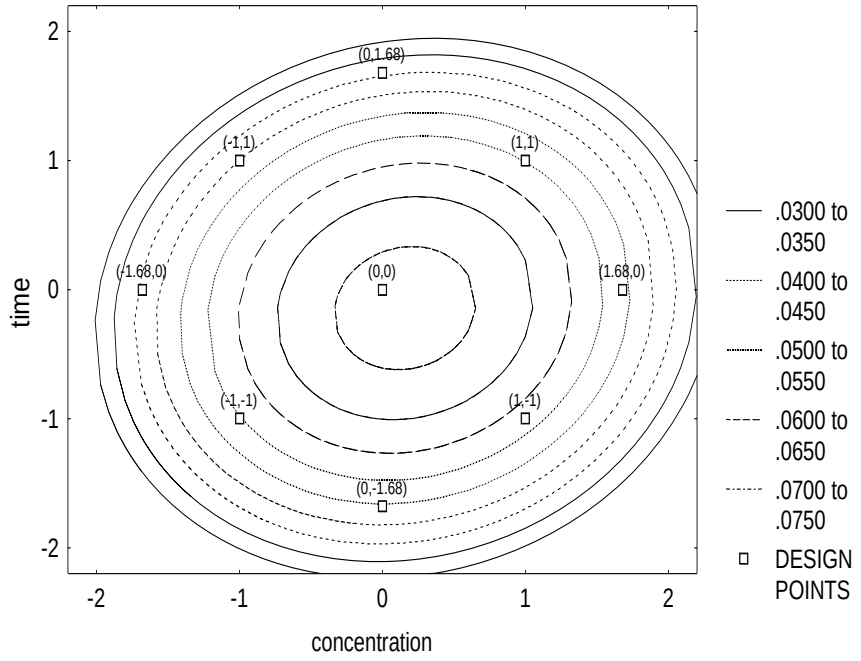

Fig. 2. Two-dimensional surface of response to activity of immobilized papain. Level lines show the enzyme activity

It must be stressed here that our assumption that papain immobilization is temperature-sensitive has not been confirmed by experiments. Adsorption of enzymes on polymer matrices is a simple method of wide applicability. It allows obtaining preparation of high enzyme load. In our case, the yield of immobilization reached the level of 35 mg/m^2 for the membrane A and 42 mg/m^2 for the membrane B. No correlation was found between the enzyme activity and the protein loading (correlation coefficients were below 0.1). However, it is too early to withdraw any conclusions from this fact and the above hypothesis should be verified by testing a large number of polymeric substrates.

4. CONCLUDING REMARKS

The statistical methods are a powerful tool in designing of experiment details and in evaluation of the data obtained. Their use allows finding the optimal conditions for adsorptive immobilization of papain on surfaces of weak-basic membranes. It has been shown that the process depends on the enzyme concentration and the exposition time. Temperature did not produce any significant effect on the yield of immobilization. A detailed analysis of the response surface allowed us to establish immobilization conditions as follows: the papain concentration in the range of 6–7 mg/cm^3 and the time of 12–14 hours. It was also found that the surface coverage reached the level of 35–40 mg/m^2.

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