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The Preparation, Characterization and Properties of Catalase Immobilized on Crosslinked Gellan

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In the present paper, the reaction of chemical immobilization of catalase on a crosslinked macromolecular carrier of a polysaccharide structure (gellan) is studied. The influence of some reaction parameters (enzyme/carrier, activator/carrier ratios, duration) on the activity of enzymatic products is analyzed. The kinetics of the biocatalytic process, stability under different pH and temperature conditions, and the inhibitors effect were studied for the immobilized enzymes.

Keywords catalase, gellan, hydrogel, enzyme immobilization

Introduction

The chemical binding of the enzymes on different carriers, especially macromolecular ones, has been studied for more than five decades. The immobilized enzymes can be used as models for *in vivo* (1, 2) enzymatic reactions, as biocatalysts with the possibility of using them several times in enzymatic reactions, as well as for detecting and treating the clinical anomalies associated with the enzymatic deficiencies (3–5).

There are various possibilities to bind an enzyme on macromolecular supports: including in hydrogels, microencapsulation, physical adsorption, and chemical binding. The last method assures a strong fixation of the enzyme on the support matrix and can be achieved by the reaction of the protein functional groups with those on the polymer carrier (amidation, esterification).

Different enzymes, belonging especially to the hydrolase's class, have been immobilized by this method (6–11).

In the present paper, the catalase immobilization on polymeric support of a polysaccharide structure, namely crosslinked gellan, is reported. The factors influencing the coupling reaction are studied and the obtained enzymatic systems are characterized

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regarding their stability at temperature and pH values; the effect of some agents with inhibiting action upon the enzyme is analyzed as well. The influence of the substratum and enzymatic product concentrations on the biocatalytical activity was also investigated.

Experimental

Materials

Catalase (Cat) is an oxidoreductase and shows catalytic action in various biological reduction–oxidation reactions. It is considered as the first defense line of hemoglobin since it catalyses the decomposition of hydrogen peroxide (a toxic metabolite for the living organisms) arising either in metabolic processes or under the action of radiations. The catalase from bovine liver ($M_w = 500.000$) has been used for experiments. The catalase's structure is presented in Scheme 1.

The gellan (Gel) is an extra-cellular heteropolysaccharide produced by *Pseudomonas elodea*. The repeating unit in the macromolecular structure consists of four saccharide residues (glucose, glucuronic acid, glucose, ramnose) (12) (Scheme 2).

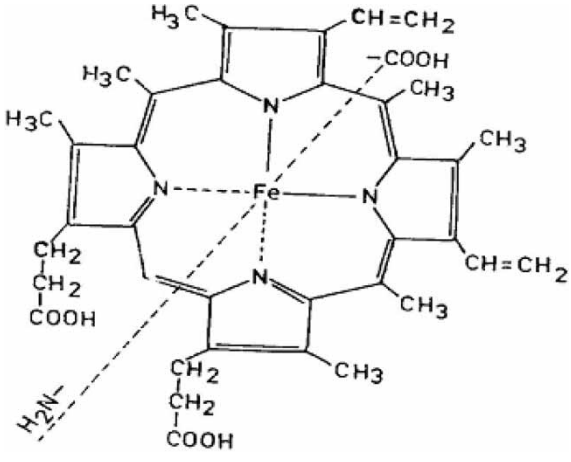
It is water soluble at a slightly basic pH and is swollen at neutral or acid pH.

In order to be used as a carrier for catalase immobilization, the gellan was previously crosslinked with epichlorohydrin in a strongly basic medium when a hydrogel with a swelling degree in water attaining 642% at equilibrium (after 2 h) has been obtained.

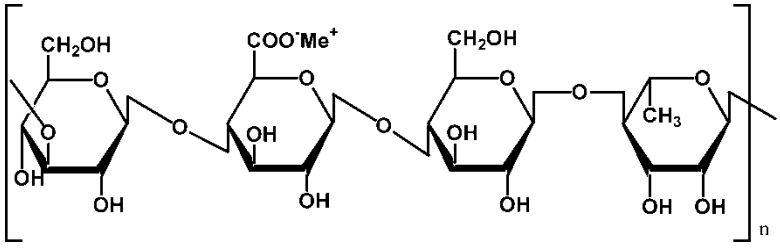
Epichlorohydrin (EPCl), used as a crosslinking agent and dicyclohexylcarbodiimide (DCCI) reagents were obtained from the Aldrich Chemical Company, Milwaukee, WI.

Gellan Crosslinking

0.5 g of gel was dissolved in 2 mL water, then a 0.25 mL 10 N NaOH solution were added and the homogenization continued for another 5 min. The required amount of epichlorohydrin was added as small portions, under continuous stirring during a 10 min period.



Scheme 1. Structure of catalase.



Scheme 2. Structure of gellan.

The resulting pasty mixture (thick gel) was poured into glass molds and kept in an oven at the temperature required for crosslinking. The obtained hydrogel was removed and immersed for 6 h in distilled water at 50°C, in order to remove the unreacted reagents.

Since the crosslinking proceeds in basic medium, the carboxyl groups of the polysaccharide are converted into salt. These groups must be restored in order to react with the enzyme and for this reason, the product requires washing with a slightly acid HCl solution (pH = 4). Therefore, the crosslinked product was suspended into a 100 mL HCl solution for 4 h, under stirring, then the suspension was centrifuged and the pH was measured. When it is still slightly acid (but higher than that of the initial HCl solution), the –COONa groups may be considered entirely converted into acid groups. Three successive washings were followed by polymer suspending into twice-distilled water, under stirring for 2 h, at 50°C until the pH value of the washing water was identical to that of the distilled water.

Finally, the hydrogel was extracted with acetone for 6 h (in a Soxhlet) in order to remove the epichlorohydrin traces and the water was dried at 40°C under vacuum; it was then grounded.

The crosslinked gellan was characterized for its swelling capacity in water by the Dogatkin method (13).

Catalase Immobilization on Crosslinked Gellan

Particles (0.2 g) of crosslinked gellan (a constant amount in all syntheses) were suspended in 20 mL of 0.5 M disodium phosphate buffer (pH = 6.5). Then, the enzymatic liquid containing the required catalase amount was added under good stirring. After stirring for 2 h, the maximum swelling degree of the hydrogel was attained and the activator dissolved in 2 mL tetrahydrofuran (THF) was added. This moment was considered as the time "zero" of the reaction. The volume was then completed to 25 mL with a phosphate buffer with the purpose of having a constant reaction volume in all experiments. The reaction was carried out at 5°C under good stirring, and the duration was that prescribed for the experimental program.

The final mixture was centrifuged for 10 min at 3000 rot/min, the supernatant layer was separated, a volume of 10 mL phosphate buffer was added and finally centrifuged for 10 min. The washing was repeated 5 times for the complete removal of the enzyme excess, dicyclohexylcarbodiimide, and the resulting dicyclohexylurea (DCCU).

The centrifuged sediment was introduced quantitatively into a flask and brought to the volume of 20 mL with 0.05 M phosphate buffer (pH = 7). The enzymatic products were kept at a temperature of 4°C.

Estimation of Enzymatic Activity

The iodometric method (14) has been applied with the purpose of evaluating the enzymatic activity.

Primarily, the method is based on the reaction of the remaining hydrogen peroxide, which has not been decomposed in a certain time of incubation in the enzyme presence. In an acid medium, the oxidation described by the following reaction proceeds:

$$H_2O_2 + 2KI + H_2SO_4 \longrightarrow 2H_2O + K_2SO_4 + I_2$$

The resulting iodine is titrated with sodium thiosulfate of a known concentration in the presence of starch as an indicator:

$$I_2 + Na_2S_2O_3 \longrightarrow 2NaI + Na_2S_4O_6$$

At the same time, a blank test without enzyme is prepared. The difference between the sodium thiosulfate volumes consumed with titration of hydrogen peroxide in the blank test and in the sample produces catalase activity.

6 mL of 0.5 M Phosphate buffer, pH = 7 was introduced in each of two 100 mL conic flasks. A 0.1 mL suspension of enzymatic product was introduced in one (after previous stirring of the flask containing the preparation in view of homogenization), and the other was intended for the flask test. In both flasks, a 0.2 mL of 3% (w/vol) H_2O_2 solution was introduced, and after a thorough stirring, they were maintained at room temperature exactly for 5 min, counted from the moment of hydrogen peroxide dropping.

The enzyme action was then stopped by adding 5 mL of a 10% (w/vol) H_2SO_4 solution. After adding an additional 5 mL of a 10% (w/vol) KI solution and a drop of 1% ammonium molybdate, the flasks were stirred vigorously.

The iodine released in reaction was titrated with a 0.02 N sodium thiosulfate solution until a straw yellow color, then 2–3 drops of a 1% starch solution were added and the titration continued until the color occurred when the starch addition completely vanished. Under the catalase action, the amount of decomposed H_2O_2 was calculated according to the relations below.

The unit of enzymatic activity of catalase is considered as the amount which decomposes $1 \cdot 10^{-3}$ mol s of H_2O_2 (0.034 mg) in a minute time. According to the reaction, 0.34 mg H_2O_2 corresponds to 1 mL of 0.02 N $Na_2S_2O_3$ solution. For the volume difference of $Na_2S_2O_3$ solution (of factor F) corresponding to the titration of the witness (V) and the sample (v), the amount of the decomposed H_2O_2 is:

$$x = (V - v) \times F \times 0.34 \text{ (mg)}$$

The amount of H_2O_2 decomposed within a minute by the enzyme from 0.1 mL enzymatic liquid was estimated by using the relation:

$$x_1 = x/5 \times 0.1$$

where 5 is the enzyme action time.

One catalase enzymatic unit decomposes 0.034 mg H_2O_2/min, which leads to the final relation for activity estimation:

$$A = x_1/0.034 = 20\ (V - v) \times F$$

Results and Discussion

The gellan was crosslinked with epichlorohydrin with the purpose to obtain a macromolecular carrier with carboxyl groups, swellable but not soluble, in aqueous solutions. The crosslinking reaction of gellan with epichlorohydrin is shown in Scheme 3.

Various network densities and swelling characteristics (Q, %) were obtained by working with different values of crosslinking process parameters. The water swelling kinetics of the crosslinked gellan under different conditions are depicted in Figure 1.

The catalase was immobilized on the carrier with a maximum swelling degree, $Q_{max} = 642\%$. The catalase coupling on crosslinked gellan was conducted in the presence of DCCI as an activator. The coupling is based on the amidation reaction between the carboxyl groups on support and the amino groups on enzyme (Scheme 4) and, possibly, on the esterification between the hydroxyl groups on support and the carboxyl groups on enzyme.

The reaction of immobilization of the catalase on synthetic macromolecular carriers (15), as well as in the case of coupling of other enzymes on polymers, was found to be influenced by the following factors: enzyme/carrier weight ratio, activator/carrier weight ratio, and coupling reaction duration.

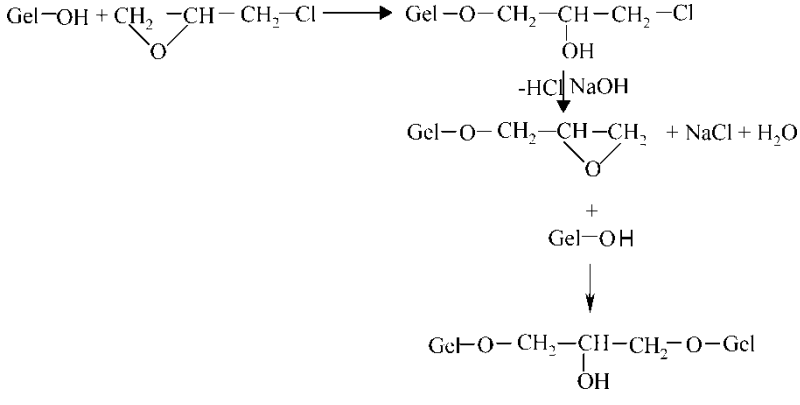
Influence of Enzyme/Carrier Ratio

The crosslinked gellan shows a high swelling capacity in water, but it is not water-soluble, so that its coupling with enzyme proceeds in a heterogeneous medium. Consequently, the diffusional process has an important role in reaction development and in attaining high coupling efficiency. Hence, the enzyme concentration in the initial reaction mixture would influence the enzymatic activity of the obtained products, which is illustrated by the results presented in Figure 2.

It is obvious that the increasing Cat/Gel ratio signifies an increase of the biocatalyst concentration in system, which would promote the intensification of enzyme diffusion inside the swollen polymer particles and, thus, the coupling yield increases.

Influence of Activator/Support Ratio

The DCCI activates the carboxyl groups of both enzyme and crosslinked gellan, and by its concentration increasing in the reaction medium, its diffusion towards these functional



Scheme 3. Reaction of gellan crosslinking with epichlorohydrin.

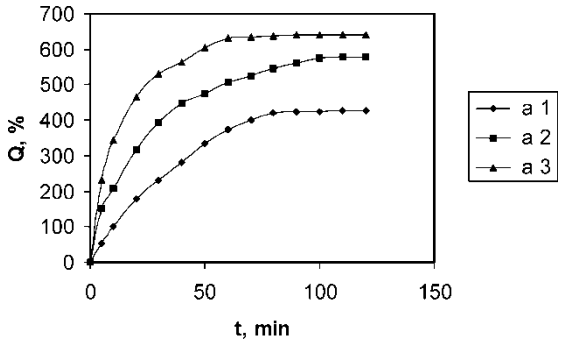


Figure 1. Swelling kinetic curves of gellan crosslinked under different reaction conditions: a1—T = 50°C, t = 3 h; a2—T = 60°C, t = 4 h; a3—T = 60°C, t = 9 h.

groups increases too. An increase in enzymatic activity of the synthesised products would result, as an effect of the increase in the amount of immobilized enzyme. As can be seen in Figure 3, this effect is exerted until a DCCI/Gel mole ratio of 2.5 above in which a tendency of activity decrease is noticed.

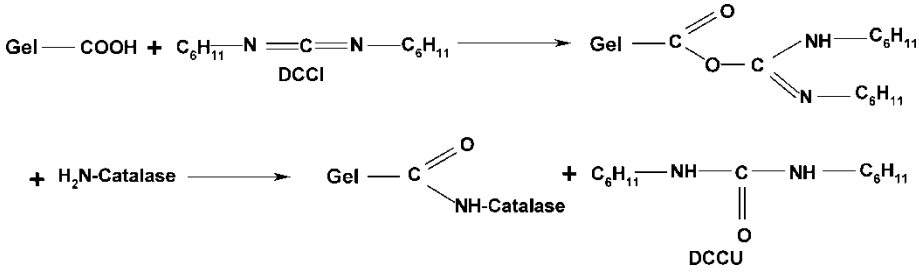
This effect is a consequence of the fact that high activator concentrations promote enzyme intermolecular condensation reactions and prevents the biocatalyst–carrier reaction. At the same time, the already immobilized enzyme could participate to cross-linking reactions, which diminish its activity. The results are in agreement with those reported in a previous study on the catalase immobilization on a synthetic copolymer poly(acrylic acid-co-vinyl alcohol) (15).

Duration

The reaction duration increased results in the enzymatic activity of the obtained products (Figure 4) increasing until a maximum is attained after 30 h.

This effect is clearly caused by the activation of an increased number of functional groups on the crosslinked gellan, which are able to couple with the enzyme. The slight decrease tendency of the enzymatic activity might be due to the side reactions in a system competing with the enzyme coupling on support (enzyme denaturation and inter- and intramolecular condensations).

Based on the presented results, one may conclude that maximum values of the enzymatic activity of the synthesised products can result when a maximum enzyme/carrier ratio, an activator/carrier mole ratio of 1.3 and reaction duration of 23 h are used.



Scheme 4. Reaction of gellan with catalase activated by DCCI.

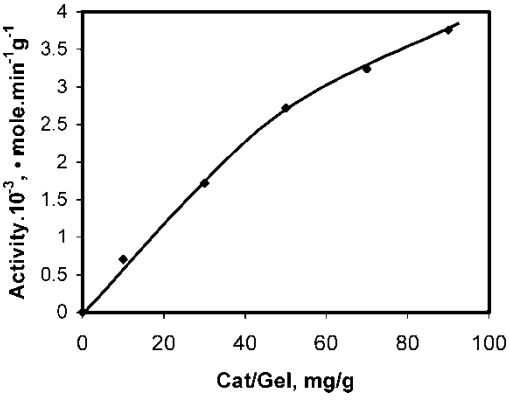


Figure 2. The enzymatic activity of coupling products vs. Cat/Gel ratio (t = 20 h; DCCI/Gel ratio = 1.3 mol/mol).

The product with a maximum enzymatic activity was analyzed regarding the kinetics of the reaction catalyzed by it, as well as the characteristic properties, of the free and immobilized enzymes were compared.

Influence of Enzymatic Product Concentration

The increase in enzyme concentration results in a proportional increase of the modification rate of the substratum, as shown in Figure 5.

With low and moderate concentrations of the enzymatic product, the process rate increases linearly. At high enzyme concentrations, the linearity is not valid, probably due to some enzyme inter- and intramolecular interactions.

Similarly to the immobilization on a synthetic macromolecular carrier (15), the immobilized catalase obeys the linearity over a wider concentration range, probably since the molecules of immobilized enzyme have a lower degree of freedom in comparison with the free enzyme. Consequently, the interaction possibilities of the protein molecules are few, which diminishes the inter- and intramolecular interactions.

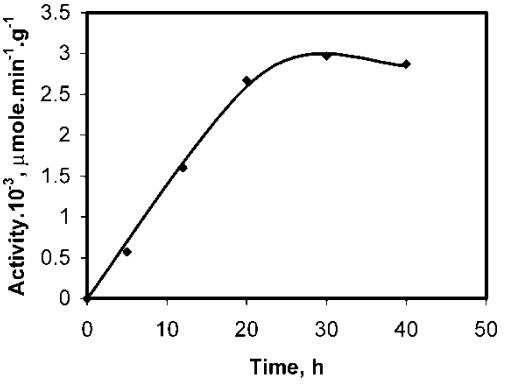


Figure 3. The enzymatic activity of coupling products vs. DCCI/Gel ratio (t = 23 h; Cat/Gel = 50 mg/g).

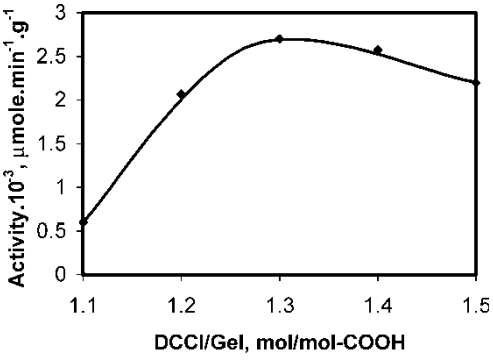


Figure 4. The enzymatic activity of coupling products versus reaction duration (Cat/Gel = 50 mg/g; DCCI/Gel = 1.3 mol/mol).

Influence of Substratum Concentration

The enzymatic catalysis first involves the formation of an intermediary complex, which can or cannot convert then into reaction products:

$$E + S \quad ES \quad E \longrightarrow \longrightarrow \quad +P$$

The mathematical modeling of this process is lead by the Michaelis-Menten equation:

$$V = V_{max} \times [S]/([S] + K_M)$$

where:

V = rate of reaction;
V_{max} = maximum rate of reaction;
[S] = molar concentration of substratum;
K_M = Michaelis constant.

The dependence of the rate of reaction on the substratum concentration is depicted in Figure 6; the Lineweaver-Burk plotting performed on its basis affords the estimation of Michaelis constant (Figure 7).

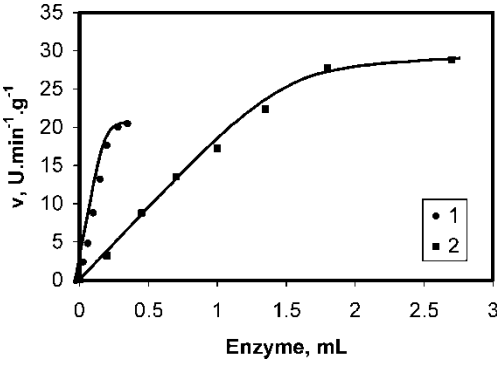


Figure 5. Influence of enzymatic preparation concentration on the reaction rate of hydrogen peroxide decomposition. 1—free enzyme; 2—immobilized enzyme.

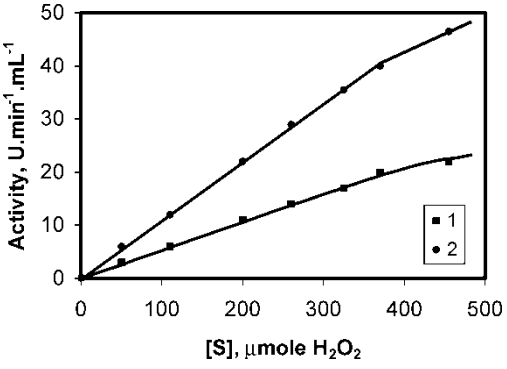


Figure 6. Variation of the enzymatic products activity vs. substratum concentration. 1—free catalase; 2—immobilized catalase (Cat/Gel = 90 mg/g; DCCI/Gel = 1.3 mol/mol; t = 23 h).

One can observe that at low H_2O_2 concentrations, the rate of reaction increases proportionally with substratum concentration. With both enzyme types, the curves are noticed to flatten at higher concentrations, which might be explained by the inhibiting effect of H_2O_2 exerted at high concentrations, as mentioned in literature (1).

The catalase immobilization on a polymeric support results in an increased value of the Michaelis constant (Figure 7), which can be noticed with enzymes immobilized on macromolecular supports. This increase signifies a lower affinity of the immobilized catalase toward the substratum and could be explained by the preventing the substratum from diffusing to the active sites of the immobilized biocatalyst; the steric hindrances and electrical barriers are responsible for this effect.

Influence of Temperature

The immobilized enzyme was also characterized in comparison with the free one as regards to the behavior under the temperature influence. The activity variation with crosslinking temperature of the two types of enzymes is depicted in Figure 8.

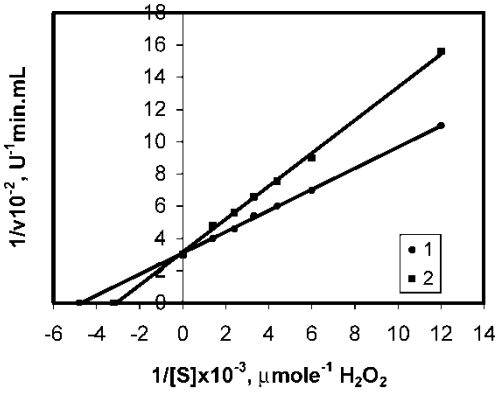


Figure 7. Estimation of Michaelis-Menten constant with free (1) and immobilized (2) catalase (Cat/Gel = 90 mg/g; DCCI/Gel = 1.3 mol/mol; t = 23 h).

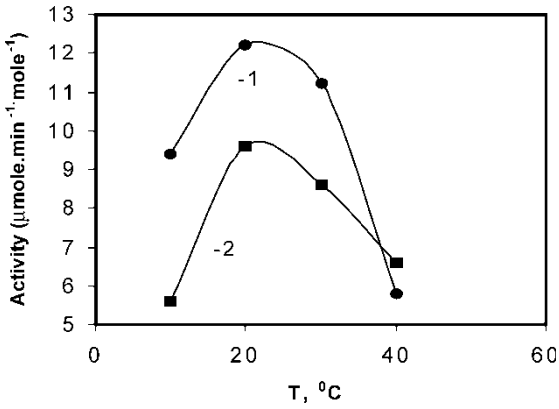


Figure 8. The activity of free (1) and immobilized (2) catalase (Cat/Gel = 90 mg/g; DCCI/Gel = 1.3 mol/mol; t = 23 h) vs. temperature.

The maximum activity of both enzymes is noticed at the temperature of 20°C, the curve shapes being similar. Obviously, the immobilized enzyme activity is permanently lower that of the free enzyme.

Higher temperatures result in an increased enzyme inactivation, as is made evident in Table 1.

It can also be noticed that the duration of enzyme exposure to temperatures under study determines the continuous decreasing in the enzyme activity.

Influence of pH

Generally, the medium pH is a significant factor influencing the enzymatic activity. The influence of this parameter upon the relative activity, estimated with respect to the maximum real activity, is shown in Figure 9.

It can be seen that in both cases (free and immobilized enzyme) the curves of relative activity variation have the same shape. It is noticeable that the maximum activity of the

Table 1
Data on thermal inactivation of the free and immobilized catalase (Cat/Gel = 90 mg/g; DCCI/Gel = 1.3 mol/mol; t = 23 h)

Catalase	Temperature (°C)	Realative activity value (%) at various durations (min) and temperatures		
		30	60	120
Free	40	100	81.33	73.33
	60	89.33	77.33	28.0
	80	9.33	0	0
Immobilized	40	100	63.16	0
	60	78.95	47.37	0
	80	26.32	21.05	0

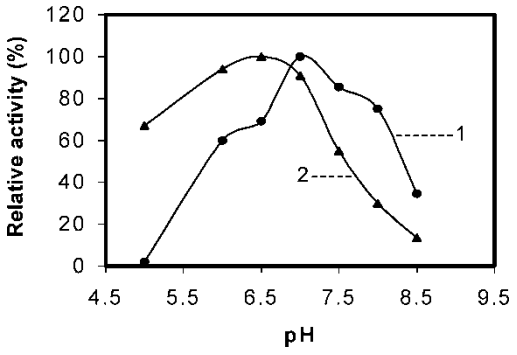


Figure 9. The relative activity of free (1) and immobilized (2) catalase (Cat/Gel = 90 mg/g; DCCI/Gel = 1.3 mol/mol; t = 23 h) vs. reaction medium pH.

immobilized enzyme shifts toward lower pH values, namely from pH = 7.5 (characteristic of the free enzyme) to pH = 6.5 (characteristic of the immobilized enzyme). Similar effects caused by the immobilized on macromolecular supports are to be found in literature regarding other enzymes (1).

Influence of Some Substances with Inhibiting Action

The catalase activity is diminished in the presence of inhibitors of both organic and mineral natures.

The sodium azide is an organic inhibitor and its influence is presented in Figure 10. A stronger action on the immobilized enzyme than on the free one can be observed.

Several metallic ions and mineral salts also have an inhibiting action on catalase. The variation of the relative activity of the enzymatic products in the presence of Cu^{2+} and Fe^{3+} (in $CaCl_2 \times 2H_2O$ and $FeCl_3 \times 6H_2O$, respectively) is illustrated in Figure 11.

A strong decrease until the complete inhibition of the enzymatic activity with increasing concentration of metallic ions can be noticed, the decrease being more severe with the free enzyme.

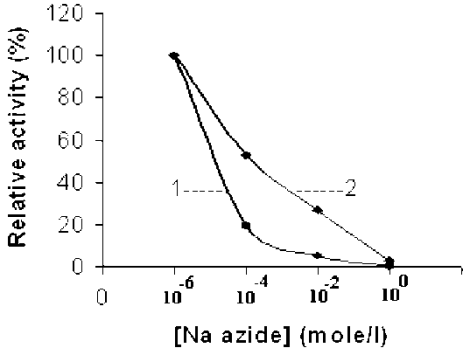


Figure 10. Influence of sodium azide on the relative activity of immobilized (1) (Cat/Gel = 90 mg/g; DCCI/Gel = 1.3 mol/mol; t = 23 h) and free catalase (2)

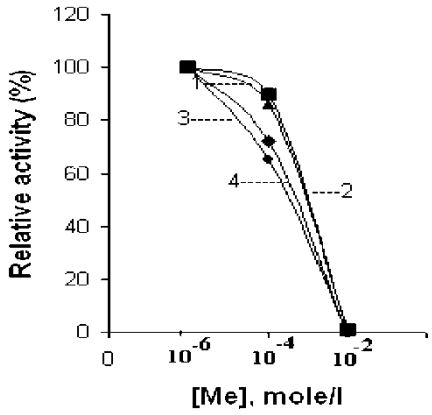


Figure 11. Influence of Cu^{2+}: 1—immobilized enzyme; 2—immobilized enzyme; 3—free enzyme and of Fe^{3+} ions; 4—free enzyme, on the catalase relative activity (the immobilization was performed under the following conditions: Cat/Gel = 90 mg/g; DCCI/Gel = 1.3 mol/mol; t = 23 h).

Conclusions

The gellan crosslinked with epichlorohydrin may be used as a carrier for the immobilization of catalase by amide or ester covalent bonds, in the presence of dicyclohexylcarbodiimide as an activator.

The coupling reaction of catalase on crosslinked gellan is influenced by the enzyme/carrier, activator/carrier ratios, and duration; the activity of the enzymatic products was found generally to increase with increasing of these parameters values.

The concentration of substratum and biocatalyst has a similar influence on the activity of enzymatic products, either both free and immobilized enzymes.

The increase in incubation temperature results in the decrease of the enzymatic activity, until inactivation, with both free and immobilized enzymes.

The pH value influences in the same manner the activity of free and immobilized enzymes, whose maximum is shifted to the pH acid range.

The substances with inhibiting action (sodium azide, Ca, Fe) diminish up to inhibition the enzymatic activity with their increasing concentration, this effect being stronger noticeably for the immobilized enzyme.

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