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Enhancement of biodegradation potential of catechol 1,2-dioxygenase through its immobilization in calcium alginate gel

Urszula Guzik *, Katarzyna Hupert-Kocurek, Ariel Marchlewicz, Danuta Wojcieszyńska

Department of Biochemistry, Faculty of and Environmental Protection, University of Silesia in Katowice, Jagiellonska 28, Katowice, Poland

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ABSTRACT

Background: In biodegradation processes free enzymes often undergo deactivation. Thus, it is very important to obtain highly stable enzymes by different methods. Immobilization allows for successful stabilization of many multimeric enzymes by increasing the rigidity of the enzyme structure. This study aimed to evaluate some environmental factors that affect catechol 1,2-dioxygenase from *Stenotrophomonas maltophilia* KB2 immobilized in alginate hydrogel. The goal of the present work was to improve the functional stability of the enzyme by increasing its structural rigidity.

Results: Immobilization yield and expressed activity were 100% and 56%, respectively. Under the same storage conditions, the activity of the immobilized enzyme was still observed on the 28th d of incubation at 4°C, whereas the free enzyme lost its activity after 14 d. The immobilized enzyme required approximately 10°C lower temperature for its optimal activity than the free enzyme. Immobilization shifted the optimal pH from 8 for the soluble enzyme to 7 for the immobilized enzyme. The immobilized catechol 1,2-dioxygenase showed activity against 3-methylcatechol, 4-methylcatechol, 3-chlorocatechol, 4-chlorocatechol, and 3,5-dichlorocatechol. The immobilization of the enzyme promoted its stabilization against any distorting agents: aliphatic alcohols, phenols, and chelators.

Conclusions: The entrapment of the catechol 1,2-dioxygenase from *S. maltophilia* KB2 has been shown to be an effective method for improving the functional properties of the enzyme. Increased resistance to inactivation by higher substrate concentration and other factors affecting enzyme activity as well as broadened substrate specificity compared to the soluble enzyme, makes the immobilized catechol 1,2-dioxygenase suitable for the bioremediation and detoxification of xenobiotic-contaminated environments.

1. Introduction

Catechol 1,2-dioxygenase from *Stenotrophomonas maltophilia* KB2 is a highly active enzyme, and for that reason it can be used for the industrial-scale production of *cis*,*cis*-muconic acid [1]. On the other hand, the environment is polluted by a lot of aromatic compounds, such as chlorophenols, cresols, or nitrophenols, which can be substrates for catechol 1,2-dioxygenases [2,3,4,5,6,7]. However, in biodegradation and industrial processes free enzymes often undergo deactivation. Thus, it is very important to obtain highly stable enzymes by different methods. One of them is immobilization, which allows for the successful stabilization of many multimeric enzymes against dissociation into subunits by increasing the rigidity of the enzyme structure, which reduces the propensity for inactivation via conformational changes and chemical inactivation [8,9,10]. Rodrigues et al. [11] suggest that the multisubunit immobilization of a multimeric enzyme allows the use of the entrapped enzyme under conditions where the free enzyme dissociates into subunits, and in this way a new enzyme conformation may present better properties than the native enzyme. Moreover, enzyme immobilization has been used for the repetitive usage of a single batch of enzyme, obtaining pure product and stopping a reaction rapidly by removing the encased enzyme from the solution [12,13]. The immobilization of enzymes in some cases leads to the partition of different compounds. If a low substrate concentration is used, immobilization leads to the condensation of the substrate in the catalytic center of the enzyme. A decrease in K_m is observed, while k_{cat}

* Corresponding author.
E-mail address: urszula.guzik@us.edu.pl (U. Guzik).
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remains unaltered. If the substrate concentration is high and for free enzyme substrate inhibition is observed, the partition effect reduces the concentration of the substrate. In this case K_m and K_i increase, whereas k_{cat} will remain unaltered [11]. It was observed that the immobilized enzymes had higher thermal stability and showed resistance against inhibition [9,13]. Among the different strategies to stabilize the enzymes, their immobilizations by entrapment in calcium alginate beads seem to be useful because it is a rapid, nontoxic, inexpensive, and versatile method. This method protects enzymes against environmental factors such as pH, temperature, oxygen, organic solvents, and chelators. Meanwhile, the disadvantages are transfer limitation, low enzyme loading, and the inactivation of immobilized multimeric enzymes by their dissociations into individual subunits [10,13,14,15].

Although free catechol 1,2-dioxygenase is very well described there is rather little information about the behavior of immobilized catechol 1,2-dioxygenases. Because of the biotransformation potential of catechol 1,2-dioxygenase from *S. maltophilia* KB2, we have attempted to improve the functional stability of the enzyme through its encapsulation in calcium alginate gel. Storage stability, as well as resistance to inhibitors of the immobilized enzyme, was determined and compared with that of the free enzyme. The knowledge gained may lead to the application of 1,2-dioxygenase from *S. maltophilia* strain KB2 in different biotechnological processes, such as bioremediation and others.

2. Materials and methods

2.1. Media and culture conditions

S. maltophilia KB2 (VTT E-113197) was cultivated in a mineral salt medium as described previously [6], in the presence of 6 mM benzoic acid. Cultures were incubated at 30°C and agitated at 130 rpm.

2.2. Preparation of the cell extracts

Cells were harvested in the late exponential growth phase and centrifuged at 4500 × *g* for 15 min at 4°C. Next, they were washed with a 50 mM phosphate buffer, pH 7.2, and resuspended in the same buffer. Cells were sonicated to prepare the cell extracts 6 × for 15 s and centrifuged at 9000 × *g* for 30 min at 4°C. The supernatant was used as a crude extract for enzyme assays and the immobilization procedure.

2.3. Gel formation

Catechol 1,2-dioxygenase was immobilized using calcium alginate. Three milliliters of the enzyme solution were suspended in 7 ml of 3% (w/v) sodium alginate prepared in 50 mM phosphate buffer solution (pH 7.2). After homogenization, the mixture was dropped into 25 ml 0.15 M $CaCl_2$ solution. Upon contact with the solution drops were gelled to form constant and defined-sized spheres (external diameter 2.0 mm), which remained in the solution under mild agitation to complete gel formation. After 1 h of incubation the beads were removed by a vacuum filtration, washed three times with a phosphate buffer solution, (pH 7.2), and stored at 4°C. These prepared alginate beads were used to analyze the properties of the immobilized enzyme.

The filtered $CaCl_2$ solution was collected for the determination of loading efficiency. Loading efficiency (%) was calculated using the following equation [16]:

$$\text{Loading efficiency}(\%) = [(C_i V_i - C_f V_f)/C_i V_i] \times 100;$$

where C_i is the initial protein concentration, V_i is the initial volume of enzyme solution, C_f is the protein concentration in the total filtrate, and V_f is the total volume of the filtrate.

Immobilization yield was defined as the difference obtained between the initial activity of the free enzyme before the immobilization and its activity obtained in the supernatant after immobilization divided by the enzyme activity before immobilization [17].

Expressed activity was calculated as the ratio of immobilized enzyme activity to the theoretical activity of the immobilized enzyme. The theoretical activity of the enzyme was calculated by subtracting the soluble enzyme units remaining after immobilization from that used for immobilization [18].

2.4. Enzyme assays

Benzoic acid was used as the inducer of catechol 1,2- dioxygenase in the growth medium. The enzymatic activity of soluble and immobilized catechol 1,2- dioxygenase was measured spectrophotometrically [6]. After the addition of the enzyme (in either free or immobilized form) vials were incubated at 30°C in a water-bath and shaken. At regular time intervals (30 s), 1 ml of aliquot was withdrawn and used to monitor the reaction progress by measuring the product *cis*,*cis*-muconic acid at 260 nm. The extinction coefficient of the oxidation product of catechol was determined as $\varepsilon_{375nm} = 16{,}800\ M^{-1}\ cm^{-1}$. One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of product per minute at 25°C. The activities of free and immobilized enzyme were expressed as specific activities (U mg^{-1} protein). The soluble and immobilized protein concentration was determined by the dye-binding procedure of Bradford, using bovine serum albumin as a standard [19]. The concentration of protein immobilized in calcium alginate gel was estimated after dissolving the beads using 1 M KOH.

2.5. pH and temperature optima of immobilized catechol 1,2-dioxygenase

The effect of pH on the immobilized enzyme activity was determined by measuring the activity at 30°C over the pH range of 2.2 to 10.0 using the following buffers: 0.05 M glycine (pH 2.2), 0.05 M phosphate-citrate (pH 3.0 to 5.0), 0.05 M Sörensen (pH 6.0 to 8.0), and 0.05 M borate (pH 9.0–10.0).

The optimum temperature was determined by assaying the enzyme activity at various temperatures (4 to 60°C) in 50 mM phosphate buffer solution (pH 7.2). The enzyme and the substrate solutions were pre-incubated, mixed, and followed by the enzymatic reaction at the same temperature.

2.6. Determination of kinetic constants of immobilized catechol 1,2-dioxygenase

The catalytic parameters (Michaelis–Menten constant, K_m, maximum velocity, V_{max}, and Hill constant, h) were calculated by measuring the initial linear rates of the enzymatic reaction after the addition of the different concentrations of the protocatechuic acid ranging from 0 to 100 μM at 30°C. Three independent measurements were carried out for each substrate concentration. K_m, V_{max}, and h were calculated based on the Hill equation.

2.7. Substrate specificity

The impact of various substituted derivatives of aromatic compounds on the enzyme activity was evaluated by incubating the immobilized enzyme with the respective aromatic compound (at 1 mM) for 3 min and assaying the activity. The dihydroxy-substituted derivatives of arene studied were 3- and 4-methylcatechol, 3- and 4-clorocatechol, 4,5- and 3,5-dichlorocatechol, and hydroquinone. The molar extinction coefficient used for the product from hydroquinone was 11,000 $M^{-1}\ cm^{-1}$ (at 320 nm) [20,21]. Catechol 1,2-dioxygenase activity for chlorinated and methylated derivatives of catechol was determined by using the procedures of Dorn and Knackmuss [22].

2.8. Activity in the presence of inhibitors

The impact of various aliphatic alcohols on the activity of free and immobilized enzyme, and the effect of phenols and chelators on the activity of immobilized enzyme were evaluated by incubating the enzyme with the respective inhibitor for 3 min and then assaying the residual activity. At regular time intervals (30 s), 1 ml of aliquots was withdrawn and used to monitor the reaction progress by measuring the product *cis*,*cis*-muconic acid. An assay of catechol 1,2-dioxygenase proceeded in the same way as in the case of non-inhibited enzyme.

The aliphatic alcohols studied were methanol, ethanol, propanol, and butanol (0.1 mM, 0.2 mM, and 0.3 mM). The phenols studied were 2-methylphenol, 3-methylphenol, 4-methylphenol, 2-chlorophenol, 4-chlorophenol, and 2,4-dichlorophenol, each at 0.1 mM, 0.2 mM, and 0.3 mM concentration. For inhibition studies EDTA, 2,2′-dipyridyl and phenathroline at 1 mM, 2 mM, and 3 mM concentration were used.

3. Results and discussion

3.1. Storage stability

Catechol 1,2-dioxygenase was immobilized in calcium alginate and the loading efficiency, the immobilization yield, and the expressed activity were 100%, 100%, and 56%, respectively. As we did not observe the presence of protein, nor was there any enzyme activity in the supernatant after immobilization, we assumed that all the enzymes were immobilized, and the decrease in its activity was connected with the influence of the entrapment in the enzyme activity. Singh et al. [23] suggest that the activity of immobilized enzyme is influenced by the binding mode. The effect of the binding mode is connected with the number of bonds formed between the carrier and the enzyme molecule, as well as the position and the nature of the bonds. It is possible that non-covalent bonds created between the enzyme and the carrier during the immobilization in calcium alginate influenced enzyme structure and activity.

One of the most important parameters to be considered during enzyme immobilization is storage stability. The stability of the soluble catechol 1,2-dioxygenase was compared with that of the immobilized enzyme by incubation in a phosphate buffer (50 mM, pH 7.2) at 4°C for 28 d. The entrapment of catechol 1,2-dioxygenase in the calcium alginate matrix caused the improvement of storage stability. Under the same storage conditions, on the 21st d of incubation the free enzyme was not active (Fig. 1), whereas the activity of the immobilized enzyme was 47.21 U mg^{-1} protein. Moreover, we still observed the activity of the entrapped enzyme on the 28th d of incubation at 4°C (1.75 U mg^{-1} protein) (Fig. 1).

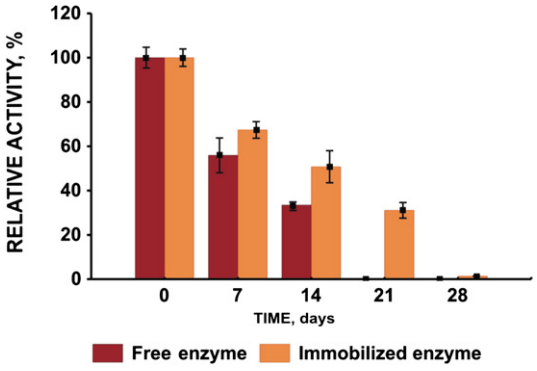

Fig. 1. Storage stabilities of free and immobilized catechol 1,2-dioxygenase from *Stenotrophomonas maltophilia* KB2. Data shown represent the average of three independent trials.

Although the immobilization of catechol 1,2-dioxygenase from strain KB2 in calcium alginate matrix improved its storage stability, the immobilized enzyme showed lower activity (128.09 U mg^{-1} protein) than the free enzyme (228.73 U mg^{-1} protein). It is suggested that the loss of the enzyme activity may be connected with distortion, dissociation of the enzyme into its individual subunits, or the barrier in the transport of hydrophobic substrates into the catalytic center [7,14,24].

Catechol 1,2-dioxygenase from strain KB2 is a homodimer, and the loss of its activity after immobilization may be connected either with the dissociation of the enzyme into its individual subunits [7,14] or the limitation of substrate (oxygen and catechol) diffusion.

3.2. Kinetic properties of immobilized catechol 1,2-dioxygenase

Temperature and pH are known to promote changes in the spatial configuration and the activity of enzymes. The pH activity profile of catechol 1,2-dioxygenase was modified by immobilization. The optimum pH of the immobilized enzyme was shifted by about a pH unit to the neutral range (Fig. 2a) compared with the free enzyme [1].

A comparison of the temperature-activity profiles of the soluble [1] and immobilized enzyme (Fig. 2b) showed that the immobilized enzyme had approximately 10°C lower optimum temperature (30°C) than that of the free enzyme (40°C). Contrary to our results, catechol 1,2-dioxygenase from *Pseudomonas putida* [9] and *Mycobacterium fortuitum* [25] required higher temperature for their optimal activities after immobilization in calcium alginate. A similar result was obtained by Wojcieszyńska et al. [26] for catechol 2,3-dioxygenase immobilized in κ-carrageenan. These results indicate the control possibility of catechol 1,2-dioxygenase activity depending on the use of the immobilized or free enzyme, which is very important for the application of this enzyme in the biodegradation processes.

We studied the behavior of the immobilized enzyme at 40°C and observed its activity over four hours. In the 4th h of the experiment the activity of the immobilized enzyme was 94.83 U mg^{-1} protein, whereas the free enzyme activity was 138.95 U mg^{-1} protein [1]. This indicates that although this temperature is not optimal for the immobilized enzyme, it shows similar behavior to the free enzyme.

In order to calculate the values of K_m, V_{max}, and h parameters, the activity of immobilized catechol 1,2-dioxygenase from *S. maltophilia* KB2 was measured at different substrate concentrations, as detailed in Materials and methods. The calculated K_m, V_{max}, and Hill constant values for the immobilized enzyme were 19.00 ± 2.21 μM, 140.79 ± 8.97 U mg^{-1} protein, and 1.68 ± 0.25, respectively (Fig. 2c) whereas K_m, V_{max}, and the Hill constant of the free enzyme were 12.18 μM, 1,218.8 U mg^{-1} protein, and 1, respectively [1].

The maximum velocity, V_{max} decreased significantly upon the immobilization of catechol 1,2-dioxygenase. Lower V_{max} after immobilization and 56% expressed activity suggest inactivation of the enzyme during the immobilization process caused by non-covalent interaction between the carrier and the enzyme or partial dissociation of the enzyme subunits [15,27]. Although a strong decrease in the immobilized enzyme activity compared to immobilized catechol 1,2-dioxygenase from *Pseudomonas putida* and free enzymes isolated from *Acinetobacter radioresistens* or *Rhodococcus opacus* was observed, our enzyme showed notably (approximately 3–70-fold) higher activity [9,28,29].

The calculated K_m for the immobilized enzyme was 37% higher than that of the free enzyme. The changes of the Michaelis constant and Hill coefficient values observed in our studies could be caused by the mass transfer limitations effect. The extent of the mass transfer is usually expressed by the effectiveness factor (EF). The calculated effectiveness factor (EF), defined as the V_{max} of the immobilized enzyme/V_{max} of free enzyme ratio was 0.12. If the EF is lower than 1.0, there is a barrier

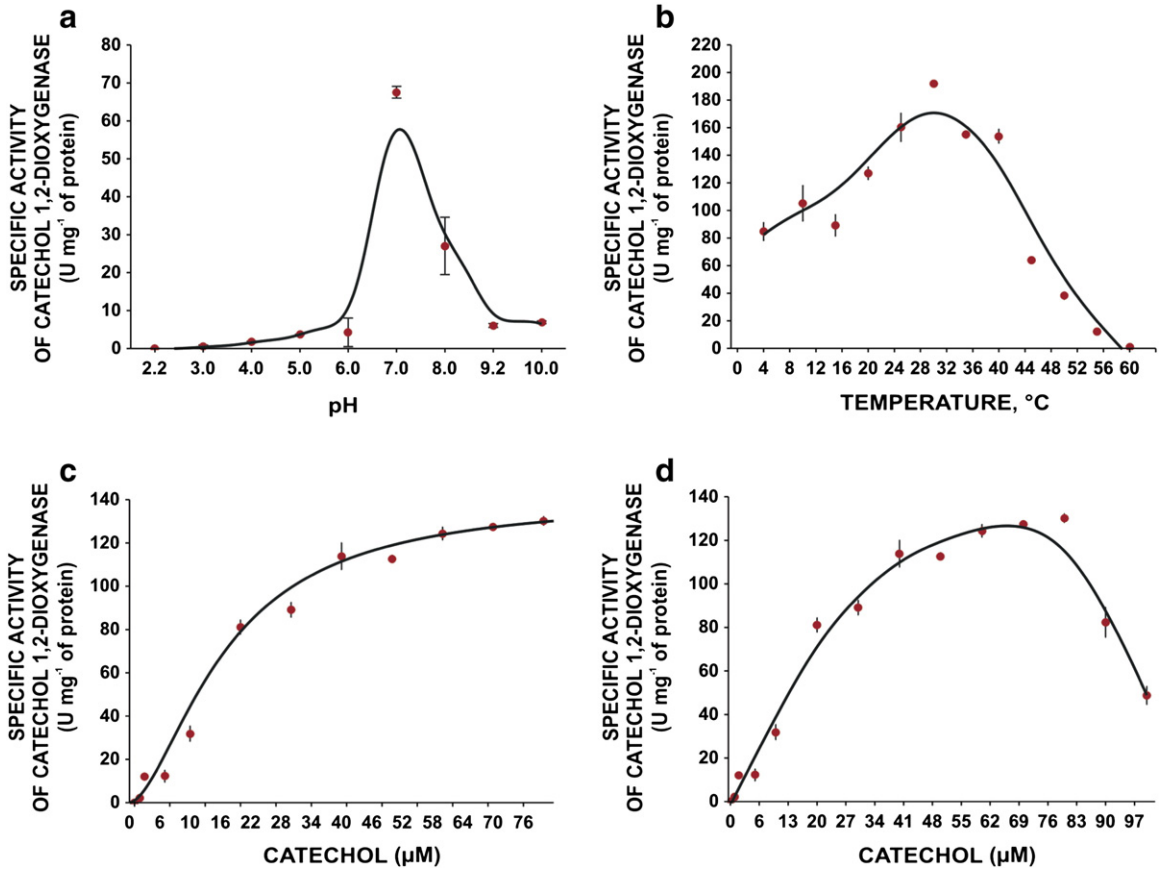

Fig. 2. Effects of pH (a), temperature (b), catechol concentration (c), and inhibition by substrate (d) on activity of immobilized catechol 1,2-dioxygenase in *Stenotrophomonas maltophilia* KB2 cell extracts. The data points represent the average of 3 independent experiments.

in the transport of substrate and product into the bead [24]. It is also reflected in the weaker inhibition by substrate. After the immobilization of catechol 1,2-dioxygenase in calcium alginate, substrate inhibition was observed at catechol concentrations above 80 μM (Fig. 2d), whereas free enzyme activity was inhibited by the substrate at concentrations above 70 μM [1]. During many industrial processes high substrate concentrations are used. This may lead to a negative effect on the enzyme activity because the substrate may produce a strong inhibition on the enzyme. The immobilization of the enzyme leads to a decrease in the local substrate concentration in the enzyme environment that may actually increase its activity [11].

3.3. Effect of immobilization on substrate specificity

Enzymes have an optimal specificity against physiological substrates. However, this limits the possibility of their use for non-natural substrates. This feature may be improved by enzyme immobilization, which provides the significant conformational changes of protein structure without losing their functions. Moreover, conformational changes in the enzyme structure which is undergone during catalysis may be distorted after immobilization. It causes altered catalytic properties of immobilized enzyme [14,30]. Catechol 1,2-dioxygenase from *S. maltophilia* KB2 shows activity against catechol, 3- and 4-methylcatechol and it was not active in the presence of chlorocatechols. It was interpreted that a halogen atom hindered oxygen attack on the aromatic ring [7]. After the immobilization of the enzyme in calcium alginate gel its activity against not only catechol and methylcatechol but also towards 3-, 4-chlorocatechol and 3,5-dichlorocatechol was observed (Table 1).

It is possible that the interaction between the carrier and the enzyme caused conformational changes which influenced enzyme activity. This may be connected with the increase in enzyme stability, since such substrates inactivate enzyme rapidly during catalysis [31]. Moreover, the partition of the substrates may increase the time of the active site saturation [11] and/or limits the local concentration of substrates, and that is why the examined immobilized dioxygenase shows a broadened substrate range. A similar effect was observed by Wojcieszyńska et al.

Table 1
Substrate specificity of immobilized catechol 1,2-dioxygenase from *Stenotrophomonas maltophilia* KB2. Data shown represent the average of three independent trials ± standard deviation.

Substrate	Relative activity of free enzyme, % [1]	Relative activity of immobilized enzyme, %
Control-catechol	100.0 ± 0.0	100.0 ± 0.00
3-Methylcatechol	11.6 ± 2.1	9.97 ± 1.68
4-Methylcatechol	23.0 ± 0.6	17.39 ± 1.71
3-chlorocatechol	0.0 ± 0.0	0.81 ± 0.00
4-Chlorocatechol	0.0 ± 0.0	1.89 ± 0.47
3,5-Dichlorocatechol	0.0 ± 0.0	0.81 ± 0.00
4,5-Dichlorocatechol	0.0 ± 0.0	0.00 ± 0.00
Hydroquinone	0.0 ± 0.0	0.00 ± 0.00

Table 2
Effect of aliphatic alcohols on the activity of free and immobilized catechol 1,2-dioxygenase from *Stenotrophomonas maltophilia* KB2. Data shown represent the average of three independent trials ± standard deviation.

Compound	Concentration (μM)	Relative activity of free enzyme, %	Relative activity of immobilized enzyme, %
None		100.0 ± 0.00	100.0 ± 0.00
Methanol	100	102.74 ± 2.90	89.21 ± 5.07
	200	74.99 ± 10.17	84.42 ± 1.63
	300	74.48 ± 6.54	84.06 ± 4.44
Ethanol	100	117.81 ± 8.62	108.23 ± 2.26
	200	108.90 ± 5.81	106.36 ± 7.92
	300	103.77 ± 15.98	104.77 ± 1.13
Propanol	100	110.44 ± 6.54	96.77 ± 5.65
	200	71.92 ± 5.81	95.97 ± 3.02
	300	0.00 ± 0.00	79.08 ± 3.63
Butanol	100	60.27 ± 11.32	88.33 ± 4.28
	200	62.67 ± 20.21	82.11 ± 6.40
	300	70.89 ± 4.36	84.59 ± 7.39

[13] for catechol 2,3-dioxygenase immobilized in calcium alginate beads.

3.4. Enzyme activity in the presence of inhibitors

Inhibitors present in the environment may interact with the enzymes inducing structure remodeling those results in enzyme inhibition. Immobilization could be the solution to this problem as it reduces the inhibitory effect of such compounds by two mechanisms. One of them is to exclude the inhibitor from the enzyme environment. The second one is to decrease the affinity of the enzyme recognition sites for the inhibitor [14].

Aliphatic alcohols and phenols substituted in the *ortho* position, which structurally mimic catechols, are known as the competitive inhibitors of catechol 1,2-dioxygenase. These compounds coordinate to the iron (III) ion at the active site of the enzyme [1,7,13,32,33].

Most of the aliphatic alcohols studied in this work influenced free and immobilized enzyme activity (Table 2). Ethanol did not affect the activity of the free and immobilized enzyme at any concentration. Propanol and butanol caused the higher inhibition of the free enzyme in comparison with the immobilized one. The inhibitory effect of these compounds may be caused by their hydrophobic interactions with the microenvironment of the enzyme. However, the weaker negative effect of the organic solvent on the entrapped enzyme was probably caused by the limited diffusion of the hydrophobic compounds by highly hydrophilic polymeric beads [11]. We suggest that the negligible effect of methanol on the enzyme activity might be connected with the steric effect. It is known that steric factors are important for the positioning of the substrate/inhibitor molecules into the catalytic cavity of catechol 1,2-dioxygenase [13,32].

In the case of 2-chlorophenol we observed a significant decrease in the activity of immobilized enzyme, whereas 2-methylphenol, 3-methylphenol, and 4-methylphenol inhibited free and immobilized catechol 1,2-dioxygenase at a similar rate (20–40%). Moreover, 4-chlorophenol and 2,4-dichlorophenol inhibited the free enzyme [1], while it did not affect the immobilized one (Table 3). Probably, lack of inhibition is connected with the steric exclusion of the inhibitor from the active site of the immobilized enzyme [11]. The protective effect of immobilization is connected with the difficult access of chlorophenol with large substituent at *para* position to the active site of the enzyme.

In our previous work we showed that catechol 1,2-dioxygenase from *S. maltophilia* KB2 is very sensitive to chelators. This is probably connected with the interaction between chelators and the iron ion in the active site. After immobilization, only 2,2′-dipyridyl still completely

Table 3
Effect of phenols on the activity of immobilized catechol 1,2-dioxygenase from *Stenotrophomonas maltophilia* KB2. Data shown represent the average of three independent trials ± standard deviation.

Compound	Concentration (μM)	Relative activity of free enzyme, % [1]	Relative activity of immobilized enzyme, %
None		100.0 ± 0.0	100.0 ± 0.00
2-Methylphenol	100	75.3 ± 6.9	78.17 ± 6.53
	200	103.9 ± 4.7	79.79 ± 9.37
	300	82.4 ± 5.4	75.20 ± 2.14
3-Methylphenol	100	79.6 ± 4.3	82.75 ± 4.94
	200	63.7 ± 0.4	77.63 ± 8.75
	300	68.3 ± 2.4	74.39 ± 8.52
4-Methylphenol	100	92.1 ± 6.5	97.04 ± 4.85
	200	70.5 ± 10.2	76.55 ± 13.63
	300	72.5 ± 0.4	73.32 ± 6.12
2-Chlorophenol	100	68.9 ± 3.2	44.07 ± 8.58
	200	67.3 ± 0.8	24.26 ± 8.00
	300	83.0 ± 6.6	8.49 ± 1.71
4-Chlorophenol	100	89.0 ± 6.8	105.12 ± 1.14
	200	87.0 ± 7.9	100.27 ± 5.72
	300	87.0 ± 5.0	99.06 ± 5.15
2,4-Dichlorophenol	100	74.0 ± 3.4	94.61 ± 3.43
	200	94.3 ± 8.1	95.82 ± 2.86
	300	70.2 ± 2.6	103.51 ± 10.29

Table 4
Effect of chelators on the activity of immobilized catechol 1,2-dioxygenase from *Stenotrophomonas maltophilia* KB2. Data shown represent the average of three independent trials ± standard deviation.

Compound	Concentration (mM)	Relative activity of free enzyme, % [1]	Relative activity of immobilized enzyme, %
None		100.0 ± 0.00	100.0 ± 0.00
EDTA	1	6.3 ± 0.1	48.01 ± 17.29
	2	5.7 ± 0.3	44.73 ± 18.87
	3	0.9 ± 0.2	37.57 ± 0.00
2,2′-Dipyridyl	1	0.1 ± 0.0	00.0 ± 0.00
	2	0.0 ± 0.0	00.0 ± 0.00
	3	0.0 ± 0.0	00.0 ± 0.00
Phenanthroline	1	1.0 ± 0.0	36.08 ± 7.17
	2	0.8 ± 0.1	9.54 ± 2.53
	3	0.0 ± 0.0	0.89 ± 0.42

inhibited the activity of the examined enzyme (Table 4). This indicates that immobilization plays the preventive role against iron chelation.

The obtained results showed that immobilized enzyme is less sensitive to the inhibition due to the slower diffusion of the inhibitor. Under these conditions the interaction of the inhibitor with the enzyme is weaker than that in the bulk solution [13,34].

The immobilization of enzymes is a notable strategy to improve their stabilities in order to realize their full potential as biocatalysts. The entrapment of the catechol 1,2-dioxygenase from *S. maltophilia* KB2 has been shown to be an effective method for improving the functional properties of the enzyme. Increased resistance to inactivation by higher substrate concentration and other factors affecting enzyme activity, as well as broadened substrate specificity compared to the soluble enzyme, makes the immobilized catechol 1,2-dioxygenase useful for environmental clean-up purposes.

Conflict of interest

All authors declare the absence of any conflict of interests including any financial, personal, or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, their work.

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