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

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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}

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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].