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Properties of catechol 2,3-dioxygenase from crude extract of *Stenotrophomonas maltophilia* strain KB2 immobilized in calcium alginate hydrogels

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ABSTRACT

In this paper we report the immobilization of catechol 2,3-dioxygenase from *Stenotrophomonas maltophilia* KB2 in alginate hydrogel with the aim of improving its functional stability by increasing structural rigidity of the enzyme. Immobilization yield and expressed activity were 49.4% and 49.4%, respectively. The storage stability of entrapped catechol 2,3-dioxygenase at 4 °C was found up to 35 days (266.3 mU/mg protein), while at 4 °C the free enzyme lost its activity within 24 h. Immobilization of dioxygenase increased the optimum temperature for activity by 10 °C, while both soluble and immobilized enzyme showed maximum activity at the same pH. The K_m, V_{max}, and Hill constant values for immobilized enzyme were 0.2 μM, 604.6 mU/mg protein, and 1.00, respectively, whereas those for the free enzyme were 46.3 μM, 1602.0 mU/mg protein, and 4.1, respectively.The immobilized catechol 2,3-dioxygenase from KB2 strain showed relatively higher activity against 3-methylcatechol, 4-methylcatechol, 4,5-dichlorocatechol, 3,5-dichlorocatechol, hydroquinone and tetrachlorohydroquinone than soluble enzyme. Immobilization of catechol 2,3-dioxygenase from KB2 strain protected the enzyme from the inhibition and enhanced its resistance to inactivation during catalysis. That makes the enzyme suitable for the bioremediation and detoxification of xenobiotic-contaminated environments.

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1. Introduction

Catechol and its derivatives are common intermediates in aerobic degradation pathways of natural and xenobiotic aromatic pollutants, which undergo ring fission through the addition of oxygen molecule by catechol 1,2- or 2,3-dioxygenases [1–8]. The catalytic characteristics of catechol dioxygenases, which catalyzes an extradiol cleavage of the aromatic ring at 2,3-(*meta*) position of catechol, have engendered much interest in this enzyme for regio- and stereoselective introduction of oxygen in a tremendous range of aromatic compounds, producing compounds that are not attainable by conventional chemical synthesis. Moreover, it has been reported to play an important role in numerous waste treatment applications [9–11]. Although this enzyme has a significant commercial potential, only few studies have been performed to develop industrial and biodegradation processes because of the low stability of the enzyme and its propensity for substrate, particularly molecular oxygen and product inhibition. Catechol 2,3-dioxygenases, typically multimeric enzymes, depend on Fe^{+2} for their catalytic action [12–16]. However, the presence of Fe(II) at the catalytic centre of these enzymes makes them more sensitive to oxygen or one of its reduced forms [17]. Oxidation of this iron inactivates them. Moreover, the iron cofactor is responsible for dioxygenase inactivation by the halocatechols that might chelate it as in the presence of catechol, and the rate of inactivation is reduced [18,19].

Immobilization allows for repetitive usage of a single batch of enzyme, obtaining pure product and stopping a reaction rapidly by removing the encased enzyme from the solution [20]. Additionally, it has been used for successful stabilization of many multimeric enzymes where increasing the rigidity of the enzyme structure reduces the propensity for inactivation via conformational changes and chemical inactivation [10,11]. For example Kalogeris et al. [11] observed higher thermal stability of quaternary structure of multimeric enzyme after its immobilization in sodium alginate. Probably it was connected with multipoint ionic interaction between enzyme and matrix [21,22]. As some researchers assume, hydrogel carriers such as alginate, carrageenan or acrylamide provide a protective hydrophilic microenvironment for the enzyme that additionally improves its stability [11,21–23]. Inactivation of immobilized multimeric enzymes by their dissociation into individual subunits due to protein concentration has also been observed [23,24]. It was shown that enzyme stability decreases with decreasing enzyme concentration [23].

In our previous works, we characterized highly active catechol 2,3-dioxygenase isolated from *Stenotrophomonas maltophilia* strain KB2 capable of degrading a variety of aromatic compounds such as

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phenol, catechol, cresols, benzoic acid, 4-hydroxybenzoic acid, protocatechuic acid, vanillic acid, and hydroquinone [4,25]. Nowadays many enzymes, among them dioxygenases, are frequently used in bioremediation as well as industrial processes. Since under process conditions those enzymes often gradually lose their activity it seems reasonable to apply a highly active enzyme for this purpose.

Because of high biotransformation potential of catechol 2,3-dioxygenase from strain KB2, we have attempted to further improve its functional stability through non-covalent immobilization (entrapment) in calcium alginate hydrogel. Although for stabilization of multimeric enzyme crosslinked enzyme aggregates (CLEA) strategy is recommended, this method can greatly alter enzyme specificity and enantioselectivity, what is not recommended for bioremediation processes [26,27]. Moreover CLEAs are not mechanically resistant and for that reason could be too soft for industrial applications. Entrapment of the biocatalysts in calcium alginate gel is well-known and most widely used in industrial application of enzymes [11,28]. It is recognized as a rapid, nontoxic, inexpensive and versatile method. This kind of immobilization protects enzyme against environmental factors such as pH, temperature, oxygen, organic solvent or chelators, but has the drawback of mass transfer limitation and low enzyme loading [29].

The purpose of the present study was the further exploration of the potential of 2,3-dioxygenase isolated from *S. maltophilia* strain KB2 (NCBI accession number DQ230920), to keep its catalytical activity for the oxygenation of various aromatic compounds. Storage and thermal 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 application of 2,3-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 was enriched in mineral salts medium (MSM), as described previously [8] in the presence of 10 mM phenol. Cultures were incubated at 30 °C and agitated at 130 rpm.

2.2. Preparation of cell extracts

Cells were harvested in the late exponential growth phase (after 15 h) and centrifuged at 4500 *g* for 15 min at 4 °C. Next, the cells were washed with 50 mM phosphate buffer, pH 7.0, and resuspended in the same buffer. Cells were sonicated 6× for 15 s and centrifuged at 9000 *g* for 30 min at 4 °C. The supernatant was used as crude extract for enzyme assays and immobilization procedure.

2.3. Gel formation

Catechol 2,3-dioxygenase was immobilized using calcium alginate. Three milliliters of enzyme solution were suspended in 7 ml of 3% (w/v) sodium alginate prepared in 50 mM phosphate buffer solution (pH 7.0). After homogenization the mixture was dropped into 25 ml 0.15 M $CaCl_2$ solution. Upon contact with the solution, the drops were gelled to form constant and defined-sized spheres (external diameter 2.0 mm), which remained in solution, under mild agitation, to complete the gel formation. After 1 h of incubation, the beads were removed by vacuum filtration, washed three times with phosphate buffer solution, (pH 7.0) and stored at 4 °C. Such prepared alginate beads were used to analyze properties of immobilized enzyme.

The filtered $CaCl_2$ solution was collected for the loading efficiency determination. Loading efficiency (%) was calculated using following equation [30]:

$$\text{Loading efficiency}(\%) = \left(\frac{(C_i V_i - C_f V_f)}{C_i V_i}\right) \times 100;$$

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

2.4. Enzyme assays

Phenol was used as the inducer of catechol 2,3- dioxygenase in the growth medium. Enzymatic activity of soluble and immobilized catechol 2,3- dioxygenase was measured spectrophotometrically [8]. After the addition of the enzyme (in either free or immobilized form), vials were incubated at 30 °C in water-bath with shaking. At regular time intervals (30 s), 1 ml aliquots were withdrawn and used to monitor the reaction progress by measuring the product 2-hydroxymuconic semialdehyde at 375 nm. The extinction coefficient of the oxidation product of catechol was determined as $\varepsilon_{375\,nm}$ = 36,000/M cm. One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of product per minute at 35 °C. Activities of free and immobilized enzyme were expressed as specific activities (U/mg protein). The soluble and immobilized protein concentration was determined by the dye-binding procedure of Bradford using bovine serum albumin as a standard [31]. The immobilized protein concentration was estimated after dissolving of the alginate beads using 1 M KOH.

2.5. pH and temperature optima of free and immobilized catechol 2,3-dioxygenase

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

The optimum temperature was determined by assaying the enzyme activity at various temperatures (4–55 °C) in 50 mM phosphate buffer solution (pH 7.4).

2.6. Determination of kinetic constants of catechol 2,3-dioxygenase

The catalytic parameters (Michaelis–Menten constant, K_m, maximum velocity, V_{max}, and Hill constant, h) for both free and immobilized enzyme were calculated by measuring the initial linear rates of the enzymatic reaction after the addition of different concentrations of catechol or methylcatechol ranging from 0 to 90 μM at 30 °C. Three independent measurements were carried out for each substrate concentration. K_m, V_{max}, and h were calculated from Hill equation using non-linear regression analysis.

2.7. Substrate specificity

Impact of various substituted derivatives of aromatic compounds on both free and immobilized enzyme activity was evaluated by incubating the enzyme with the respective aromatic compound for 3 min and assaying the activity. Dihydroxy-substituted derivatives of arene studied were 3- and 4-methylcatechol, 4,5- and 3,5-dichlorocatechol, hydroquinone and tetrachlorohydroquinone at 1 mM concentration. The molar extinction coefficient used for the product from 3-methylcatechol was 13,800/M cm (at 388 nm), from 4-methylcatechol was 28,100/M cm (at 382 nm) [32], from 3,5-dichlorocatechol was 10,000/M cm (at 337 nm)

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