Pol. J. Environ. Stud. Vol. 22, No. 4 (2013), 1219-1225

Original Research

Influence of the Entrapment of Catechol 2,3-Dioxygenase in κ-Carrageenan on Its Properties

Danuta Wojcieszyńska, Katarzyna Hupert-Kocurek, Urszula Guzik*

Department of Biochemistry, Faculty of Biology and Environmental Protection, University of Silesia in Katowice, Jagiellonska 28, 40-032 Katowice, Poland

Received: 3 September 2012
Accepted: 5 March 2013

Abstract

Microbial extradiol dioxygenases have a great potential in bioremediation, but their structure is very sensitive to various environmental and chemical agents. Immobilization techniques make the enzyme properties' improvement possible. This is the first report of the usage of κ-carrageenan as a matrix for the immobilization of catechol 2,3-dioxygenase. The storage stability of entrapped catechol 2,3-dioxygenase from *Stenotrophomonas maltophilia* KB2 in κ-carrageenan hydrogel at 4ºC was found up to 14 days, while the free enzyme lost its activity within 24 hours. The immobilization of dioxygenase decreased the optimum temperature by 10ºC, while both soluble and immobilized enzyme showed similar pH properties. The K_m, V_{max}, and Hill constant values for the immobilized enzyme were 0.17 μM, 106.68 mU, and 1.00, respectively. The immobilized catechol 2,3-dioxygenase showed higher activity against 3-methylcatechol, hydroquinone, and tetrachlorohydroquinone than the soluble enzyme. Immobilization of catechol 2,3-dioxygenase protected the enzyme from inhibition and enhanced its resistance to inactivation during catalysis.

Keywords: immobilization, biodegradation, κ-carrageenan, *Stenotrophomonas*, catechol 2,3-dioxygenase

Introduction

Catechol 2,3-dioxygenase of *Stenotrophomonas maltophilia* KB2 belongs to the single-ring substrate subfamily of the extradiol dioxygenase, but it is distinguished by high activity [1]. This property predisposes its usage in biodegradation processes. However, this kind of enzyme depends on Fe^{+2} for activity [2-6], and it is very sensitive to oxidative agents [7]. It is known that immobilization can stabilize multimeric enzymes. Increased rigidity of the enzyme structure leads to reduction of chemical inactivation [8].

Over the last decades, various methods including adsorbing enzymes into a porous support by physical adsorption, covalently attaching enzyme onto activated surface, cross-linking enzymes onto crystals, and entrapping enzymes into a polymer matrix have been explored to immobilize the enzymes [9]. Entrapment can be defined as a physical restriction of enzymes within a confined space or network. Gelation of the polyionic polymers by the addition of multivalent counter-ions is a simple, cheap, and easy method of biocatalyst entrapment [10]. One of the most widely used immobilization matrices is κ-carrageenan, linear, sulfated polysaccharide extracted from numerous seaweeds species, mainly from the *Rhodophyceae* family [11, 12]. The primary structure is made up of alternating α(1,3)-D-galactose-4-sulphate and β(1,4)-3,6-anhydro-D-galactose residues. κ-Carrageenan forms a gel triggered by reduction in temperature and/or through ionic interactions [11, 13].

So far catechol 2,3-dioxygenases have been immobilized only on BrCN-activated Sepharose 4B [14] or glyoxyl agarose [15]. Our previous work showed enhanced resis-

*e-mail: urszula.guzik@us.edu.pl

tance to inactivation during catalysis, increasing in stability and broadening substrate specificity after entrapment of catechol 2,3-dioxygenase in calcium alginate [16]. However, this matrix is characterized by low mechanical resistance. The present paper deals with the immobilization of catechol 2,3-dioxygenase of *Stenotrophomonas maltophilia* KB2 in κ-carrageenan as well as with the comparison of enzyme properties after its immobilization in κ-carrageenan and calcium alginate gel. To our knowledge, this is the first report of the usage of κ-carrageenan as a matrix for the immobilization of catechol 2,3-dioxygenase.

Experimental Procedures

Media and Culture Conditions

Stenotrophomonas maltophilia KB2 (VTT E-113197) was enriched in mineral salts medium (MSM), as described previously [17] in the presence of 10 mM phenol. Cultures were incubated at 30ºC and agitated at 130 rpm.

Preparation of Cell Extracts

Cells were harvested in the late exponential growth phase and centrifuged at 4,500 g for 15 min at 4ºC. Next, the cells were washed with 50 mM phosphate buffer, pH 7.0, and resuspended in the same buffer. Cell extracts were sonicated 6 × for 15 s and centrifuged at 9,000 x g for 30 min at 4ºC. The supernatant was used as crude extract for enzyme assays and immobilization procedure.

Gel Formation

Catechol 2,3-dioxygenase was immobilized with the use of κ-carrageenan. κ-Carrageenan was prepared in 50 mM phosphate buffer solution (pH 7.0) and heated to 45ºC. Three milliliters of the crude extract were suspended in 7 ml of 2% (w/v) κ-carrageenan. After homogenization the mixture was dropped into 25 ml 0.3 M KCl solution. Upon contact with the solution, the drops were gelled to form constant and defined-size spheres (external diameter 3.0 mm), which remained in the solution, under mild agitation, to complete the gel formation. After 1 h of incubation, the beads were removed, washed three times with the phosphate buffer solution (pH 7.0), and stored at 4ºC. The κ-carrageenan beads prepared this way were used to analyze the properties of the immobilized enzyme.

Enzyme Assays

Phenol was used as the inducer of catechol 2,3-dioxygenase. Enzymatic activity of soluble and immobilized catechol 2,3-dioxygenase was measured spectrophotometrically [16, 17]. After the addition of the enzyme (in either free or immobilized form), vials were incubated at 30ºC in water-bath with shaking. At certain time intervals, 1 ml aliquots were withdrawn and used to monitor the reaction's progress by measuring the product 2-hydroxymuconic semialdehyde at 375 nm. One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of product per minute at 35ºC. The soluble and immobilized protein concentration was determined by the dye-binding procedure of Bradford using bovine serum albumin as a standard [18].

pH and Temperature Optima of Free and Immobilized Catechol 2,3-Dioxygenase

The effect of pH on enzyme activity was determined by measuring the activity at 30ºC over the pH range of 2.2 to 12.0 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 9.0-12.0).

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

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 ranging from 0 to 7 μM at 30ºC. Three independent measurements were carried out for each substrate concentration. K_m, V_{max}, and h were calculated based on Hill equation.

Substrate Specificity

The 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-methylphenol, 4,5- and 3,5-dichlorophenol, hydroquinone, and tetrachlorohydroquinone at 1 mM concentration.

The molar extinction coefficient used for the product from 3-methylcatechol was 13,800 $M^{-1}\cdot cm^{-1}$ (at 388nm), from 4-methylcatechol was 28,100 $M^{-1}\cdot cm^{-1}$ (at 382 nm) [19], from 3,5-dichlorocatechol was 10,000 $M^{-1}\cdot cm^{-1}$ (at 337 nm) [20, 21], from 4,5-dichlorocatechol was 36,000 $M^{-1}\cdot cm^{-1}$ (at 375 nm), from hydroquinone was 11,000 $M^{-1}\cdot cm^{-1}$ (at 320 nm) [22, 23], and from tetrachlorohydroquinone was 11,000 $M^{-1}\cdot cm^{-1}$ (at 320 nm).

Activity in the Presence of Inhibitors

The impact of various phenols, aliphatic alcohols, sodium azide, hydrogen peroxide, and copper (II) sulphate on both free and immobilized enzyme activity was evaluated by incubating the enzyme with the respective inhibitor for 3 min and then assaying the residual activity. The phenols studied were 2-methylphenol, 3-methylphenol,

2-chlorophenol, 4-chlorophenol, and 2,4-dichlorophenol at 100 μM, 200 μM, and 300 μM concentrations. Aliphatic alcohols studied were methanol, ethanol, propanol, and butanol (100 μM, 200 μM, and 300 μM). For inhibition studies sodium azide and hydrogen peroxide at 100 μM, 200 μM, and 300 μM, and copper (II) sulphate at 100 μM, 150 μM, and 200 μM concentrations were used.

Results and Discussion

Storage Stability

The stability of an enzyme is of significant importance for scheduling its application in a particular reaction. Generally, if the enzyme is in solution, it is not stable during storage and its activity is gradually reduced. Catechol 2,3-dioxygenases are especially sensitive enzymes to oxidation [7]. The stability of the free and immobilized catechol 2,3-dioxygenase in κ-carrageenan was determined after storage of the preparations in the phosphate buffer (50 mM, pH 7.0) at 4ºC for a predetermined period. It was observed that the free enzyme loses 99.95% of its activity within 24 hours [16], while activity of the immobilized enzyme in κ-carrageenan after 14 days of incubation at 4ºC was 2.5% of the initial rate. The higher stability observed for the immobilized catechol 2,3-dioxygenase could be attributed to the prevention of auto oxidation of the enzyme. However, catechol 2,3-dioxygenase immobilized in alginate matrix represented still 50% of the initial rate after 35 days [16]. Therefore, calcium alginate seems to be a better matrix for immobilization of catechol 2,3-dioxygenase.

Although entrapment of catechol 2,3-dioxygenase from strain KB2 into κ-carrageenan matrix improved its storage stability, the immobilized enzyme showed lower activity than the free enzyme. The same effect was observed after immobilization dioxygenase in calcium alginate [16]. Catechol 2,3-dioxygenase seems to dissociate into individual subunits during immobilization. Dissociation of enzyme oligomers is a common mechanism of multimeric enzyme inactivation [24]. The specific activity of the immobilized enzyme was 276.90 $mU \cdot mg^{-1}$ of protein, which represented approximately 40% of the native enzyme activity. Nevertheless, the immobilized enzyme's activity is still comparable or higher than those observed by other authors for free catechol 2,3-dioxygenases [25-27].

Kinetic Properties of Free and Immobilized Catechol 2,3-Dioxygenase

Environmental factors that affect an enzyme's reactions include temperature and pH. pH is one of the most important factors influencing not only the side groups of amino acid dissociation in the protein structure, but also the solution chemistry of the insoluble support. Small changes in pH and ionic strength of medium may cause denaturation and deactivation of the enzyme. Immobilization of the enzyme is likely to result in conformational changes of the enzyme resulting in optimum variations [9].

The effect of pH on the activity of the free and the immobilized catechol 2,3-dioxygenase in κ-carrageenan was assayed in the pH range 2.2-12.0 (Fig. 1a). As shown in the mentioned figure, the maximum activity for free and immobilized in κ-carrageenan enzyme was observed at pH 8.0. The same result was observed after immobilization of catechol 2,3-dioxygenase in the alginate matrix [16].

Comparison of the temperature-activity profiles (Fig. 1b) showed that the immobilized enzyme in κ-carrageenan had approximately 10ºC and 20ºC lower optimum temperature than the free one immobilized in the alginate matrix [16], respectively. These results indicate control possibility

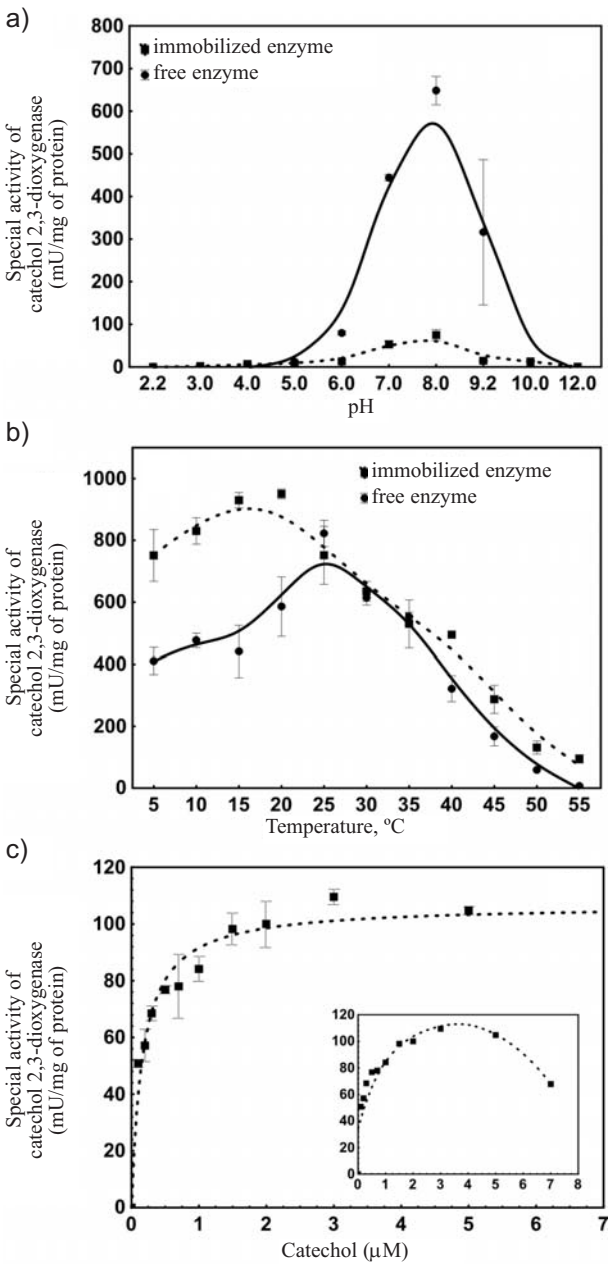

Fig. 1. Effects of pH (a) and temperature (b) on free and immobilized catechol 2,3-dioxygenase activity and influence of catechol concentration on immobilized enzyme (c) from *Stenotrophomonas maltophilia* KB2 cell extracts. The data points represent the average of 3 independent experiments.

of catechol 2,3-dioxygenase activity depending on use of the immobilization matrix, which is very important for the application of this enzyme on a commercial scale.

In order to calculate values of K_m, V_{max}, and h parameters, the activity of immobilized in κ-carrageenan and free catechol 2,3-dioxygenase from *Stenotrophomonas maltophilia* KB2 was measured at different substrate concentrations as detailed in *Experimental procedures*. The calculated K_m, V_{max}, and Hill constant values for the free enzyme were higher [16] than for the immobilized enzyme, amounting to 0.17 μM, 106.68 mU, and 1.00, respectively (Fig. 1c). These results indicate significantly higher affinity of the enzyme to substrate after immobilization in κ-carrageenan than in the case of the free enzyme. The same effect was observed during immobilization of enzyme in calcium alginate [16]. It is supposed that the changes in affinity of enzyme to substrate are connected with the loss of cooperation between subunits [24]. Change of Hill constant value also confirms the probability mentioned above. The mechanism of retaining enzyme activity after decomposition of an enzyme has to be explained because most often dissociation of the enzyme leads to their inactivation.

Decrease in K_m values also was observed by Kara and co-workers [28] for urease immobilized in alginate/chitosan and poly(acrylamide-co-acrylic acid)/κ-carrageenan. These authors suggest that this result may be attributed to electrostatic interactions between the substrate and polymeric matrix [28].

Both free and immobilized enzymes in alginate and κ-carrageenan matrix showed, in the same manner, negative deviations at high catechol concentrations (above 90 μM, 30 μM [16], and 7 μM, respectively), which could be attributed to the classical substrate inhibition. The same effect was observed by Dhulster et al. [29] and Fernandez-Lafuente et al. [15].

Effect of Immobilization on Substrate Specificity

Immobilization most often may produce some distortions on the active site, reducing the overall mobility of the protein groups [24]. Most of the known extradiol dioxygenases characterize narrow substrate specificities and regioselectivities [30, 31]. This attribute is used commercially in the pharmaceutical and chemical industries. However, use of dioxygenases in bioremediation processes requires enzymes with broad specific activity. In our previous work we observed that catechol 2,3-dioxygenase from KB2 strain showed broader substrate specificity after immobilization in calcium alginate [16]. In this study it was found that the immobilized enzyme in κ-carrageenan showed relatively higher activity (Table 1) than its free form against 3-methylcatechol, hydroquinone, and tetrachlorohydroquinone, but generally it was weaker than for enzymes immobilized in alginate matrix [16]. Broader specific activity of enzyme may be due to stabilization of immobilized enzyme and increasing resistance of enzyme on substrate toxicity [14, 32]. It is known that the specific structure of catechol 2,3-dioxygenase influences the limitation of local concentrations of the substrates, and that is why examined immobilized dioxygenase showed such a broad range of substrate specificity [16, 32].

Table 1. Catechol 2,3-dioxygenase substrate specificity immobilized in κ-carrageenan beads. Data shown represent the average of three independent trials±standard deviation.

Substrate	Activity of immobilized enzyme in κ-carrageenan beads, %
Control – catechol	100±1.85
3-methylcatechol	12.45±1.09
4-methylcatechol	60.72±0.65
4,5-dichlorocatechol	1.38±0.22
3,5-dichlorocatechol	0.86±0.56
Hydroquinone	3.00±0.54
Tetrachlorohydroquinone	1.69±0.43

Enzyme Activity in the Presence of Inhibitors

Little information is available on immobilized extradiol dioxygenases activity in the presence of various inhibitors. According to our knowledge, only Iwaki and Nozaki [14] have shown the influence of hydrogen peroxide on the activity of immobilized catechol 2,3-dioxygenase from *Pseudomonas arvilla*. Due to that reason we researched the influence of known extradiol dioxygenase inhibitors on catechol 2,3-dioxygenase from KB2 strain.

In the presence of sodium azide we observed inhibition of both free and immobilized enzymes (Table 2). This compound is a potential inhibitor of catechol 2,3-dioxygenase, which may be explained in two possible ways: by coordination of Fe^{+2} ions in active site of enzyme or/and by disruption of electron flow during aromatic ring cleavage. However, the level of inhibition depends on the kind of used matrix. Inhibition of enzyme immobilized in κ-carrageenan was stronger than in case of free enzyme (Table 2), whereas after immobilization of enzyme in calcium alginate the activity was higher than free enzyme [16].

Sensitivity to oxygen or one of its reduced forms of catechol 2,3-dioxygenase is connected with the fact that each of four catechol 2,3-dioxygenase subunits contains an essential iron atom that must be in the Fe(II) oxidation state [7]. Our previous research showed the protective effect of immobilization in calcium alginate against hydrogen peroxide [16]. Unfortunately, we observed only the slightly protective effect of immobilization of enzyme in κ-carrageenan (Table 2). Probably the differences of enzyme activity after immobilization in various matrices resulted from the dissimilarity in gel structure, which caused another inhibitor diffusion velocity.

Immobilized enzyme in κ-carrageenan also was sensitive to copper (II) sulphate as an inhibitor (Table 2). Probably a different effect of copper (II) sulphate on the

Table 2. Influence of sodium azide, hydrogen peroxide, and cooper (II) sulphate on immobilized catechol 2,3-dioxygenase activity. Data shown represent the average of three independent trials±standard deviation.

Compound	Concentrations (μM)	Activity of immobilized enzyme in κ-carrageenan beads, %
None		100±1.01
Sodium azide	100	27.10±2.35
	200	23.18±0.55
	300	19.27±1.36
Hydrogen peroxide	100	10.05±0.41
	200	6.80±0.49
	300	4.78±0.53
Copper (II) sulphate	100	2.92±0.2
	150	3.94±0.82
	200	2.18±0.29

activities of free and immobilized enzyme in κ-carrageenan is connected with the interaction of the sulphate group in the copper (II) sulphate molecule with the hydroxyl surface of the matrix, which may modify the conformation of the gel's structure and thus may result in greater accessibility of cooper ions to enzyme molecules. A similar effect of cooper citrate on immobilized acid phosphatase was observed by Huang and Shindo [33].

Aliphatic alcohols and phenols, which structurally mimic catechols, are known as competitive inhibitors of catechol 2,3-dioxygenase because they coordinate to the iron (II) ion in the enzyme active site [3].

Aliphatic alcohols caused higher inhibition of the free enzyme in comparison with the immobilized enzyme. Methanol and ethanol in concentrations of 100 μM affected strongly the immobilized enzyme in κ-carrageenan matrix (Table 3). A similar effect was observed after immobilization of enzyme in calcium alginate [16]. This confirms that the significant effect of low molecular weight alcohols on enzyme activity might be connected with the steric effect [3]. Moreover, methanol as a smaller molecule may function as a ligand of ferrous ion in the enzyme active site. Butanol used as an inhibitor showed a stronger effect than propanol and ethanol, both immobilized enzyme in calcium alginate and κ-carrageenan gel. It is connected with its higher hydrophobicity [3].

Most of the studied phenols affected free and immobilized enzyme activity at all tested concentrations (Table 3). Our previous work on catechol 2,3-dioxygenase from KB2 strain has shown that 4-chlorophenol and 2,4-dichlorophenol significantly inhibited the activity of free enzymes as well as the enzyme immobilized in calcium alginate [16]. After immobilization of enzyme in κ-carrageenan inhibition of catechol 2,3-dioxygenase was not observed. Moreover, it showed higher activity toward these substrates

Table 3. Influence of phenol and aliphatic alcohols on immobilized catechol 2,3-dioxygenase activity. Data shown represent the average of three independent trials±standard deviation.

Compound	Concentration (μM)	Activity of immobilized enzyme in κ-carrageenan beads, %
None		100±1.01
2-methylphenol	100	65.05±2.80
	200	64.12±11.22
	300	55.09±6.74
3-methylphenol	100	53.24±5.40
	200	47.68±2.80
	300	41.02±4.18
2-chlorophenol	100	86.46±5.40
	200	83.56±12.92
	300	75.93±5.90
4-chlorophenol	100	142.60±6.74
	200	102.78±17.72
	300	98.15±7.29
2,4-dichlorophenol	100	221.01±19.69
	200	197.45±16.68
	300	173.61±5.93
Methanol	100	36.28±5.22
	200	33.09±3.07
	300	28.96±0.76
Ethanol	100	60.67±5.87
	200	44.83±3.84
	300	38.32±4.44
Propanol	100	92.42±6.23
	200	63.40±2.36
	300	50.67±7.74
Butanol	100	55.60±6.05
	200	51.15±2.55
	300	37.45±1.27

(Table 3). However, an explanation of these results requires further examination, as differences in answers of enzyme immobilized in various matrices may turn out to be useful in both environmental and industrial applications of catechol 2,3-dioxygenase.

Conclusions

Entrapment of the catechol 2,3-dioxygenase in κ-carrageenan turned out to be an effective method of protection

against very toxic *para* substituent chlorophenols. This is important since these compounds exist together with other arenes, which are substrates for catechol 2,3-dioxygenase. It should be emphasized that the differences in temperature optimum and reaction on inhibitors' effect between the enzymes immobilized in κ-carrageenan and alginate may contribute to wider usage of this enzyme in bioremediation. The construction of biopreparate contains a mix of immobilized enzymes in different matrices, which seems to be the best way for improving degradation potential of catechol 2,3-dioxygenase.

Acknowledgements

This work was supported by The Polish Ministry of Science and Higher Education (N N305 212337).

Anna Jankowska and Piotr Jaroszek are acknowledged for their excellent technical assistance.

References

1. WOJCIESZYŃSKA D., HUPERT-KOCUREK K., GREŃ I., GUZIK U. High activity catechol 2,3-dioxygenase from the cresols-degrading *Stenotrophomonas maltophilia* strain KB2. Int. Biodeter. Biodegr. **65**, 853, **2011**.
2. QUE L., WIDOM J., CRAWFORD R. L. 3,4-Dihydroxyphenylacetate 2,3-dioxygenase. A manganese (II) dioxygenase from *Bacillus brevis*. J. Biol. Chem. **256**, 10941, **1981**.
3. BERTINI I., BRIGANTI F., SCOZZAFAVA A. Aliphatic and aromatic inhibitors binding to the active site of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2. FEBS Lett. **242**, 56, **1994**.
4. KASCHABEK S. R., KASBERG T., MULLER D., MARS A. E., JANSSEN D. B., REINEKE W. Degradation of chloroaromatics: purification and characterization of a novel type of chlorocatechol 2,3-dioxygenase of *Pseudomonas putida* GJ31. J. Bacteriol. **180**, 296, **1998**.
5. MILO R. E., DUFFNER F. M., MULLER R. Catechol 2,3-dioxygenase from the thermophilic, phenol-degrading *Bacillus thermoleovorans* strain A2 has unexpected low thermal stability. Extremophiles **3**, 185, **1999**.
6. PALANIANDAVAR M., MAYILMURUGAN R. Mononuclear nan-heme iron (III) complexes as functional models for catechol dioxygenases. CR Chimie **10**, 366, **2007**.
7. HASSETT D. J., OCHSNER U. A., GROCE S. L., MA J.-F., LIPSCOMB J. D. Hydrogen peroxide sensitivity of catechol 2,3-dioxygenase: a cautionary note on use of xylE reporter fusions under aerobic conditions. Appl. Environ. Microbiol. **66**, 4119, **2000**.
8. BRIGANTI F., PESSIONE E., GIUNTA C., MAZZOLI R., SCOZZAFAVA A. Purification and catalytic properties of two catechol 1,2-dioxygenase isozymes from benzoate-grown cells of *Acinetobacter radioresisten*. J. Protein. Chem. **19**, 709, **2000**.
9. TÜMTÜRK H., KARACA N., DEMIREL G., SAHIN F. Preparation and application of poly(N,N-dimethylacrylamide-co-acrylamide) and poly(N-isopropylacrylamide-co-acrylamide)/κ-Carrageenan hydrogels for immobilization of lipase. Int. J. Biol. Macromol. **40**, 281, **2007**.
10. SHAFEI M. S., ALLAM R. F. Production and immobilization of partially purified lipase from *Penicillium chrysogenum*. Malays. J. Microbiol. **6**, 196, **2010**.
11. BELYAEVA E., VALLE D. D., PONCELET D. Immobilization of α-chymotrypsin in κ-carrageenan beads prepared with the static mixer. Enzyme Microb. Technol. **34**, 108, **2004**.
12. NOBRE T. M., DE SOUSA E SILVA H., FURRIEL R. P. M., LEONE F. A., MIRANDA P. B., ZANIQUELLI M. E. D. Molecular view of the interaction between ι-carrageenan and a phospholipid film and its role in enzyme immobilization. J. Phys. Chem. **113**, 7491, **2009**.
13. GIRIGOWDA K., MULIMANI V. H. Hydrolysis of galacto-oligosaccharides in soymilk by κ-carrageenan-entrapped α-galactosidase from *Aspergillus oryzae*. World J. Microbiol. Biotechnol. **22**, 437, **2006**.
14. IWAKI M., NOZAKI M. Immobilization of metapyrocatechase and its properties in comparison with the soluble enzyme. J. Biochem. **91**, 1549, **1982**.
15. FERNANDEZ-LAFUENTE R., GUISAN J. M., ALI S., COWAN D. Immobilization of functionally unstable catechol 2,3-dioxygenase greatly improves operational stability. Enzyme Microb. Technol. **26**, 568, **2000**.
16. WOJCIESZYŃSKA D., HUPERT-KOCUREK K., JANKOWSKA A., GUZIK U. Properties of catechol 2,3-dioxygenase from crude extract of *Stenotrophomonas maltophilia* strain KB2 immobilized in calcium alginate hydrogels. Biochem. Eng. J. **66**, 1, **2012**.
17. WOJCIESZYŃSKA D., GUZIK U., GREŃ I., PERKOSZ M., HUPERT-KOCUREK K. Induction of aromatic ring - cleavage dioxygenases in *Stenotrophomonas maltophilia* strain KB2 in cometabolic systems. World J. Microbiol. Biotechnol. **27**, 805, **2011**.
18. BRADFORD M. M. A rapid and sensitive method of the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248, **1976**.
19. BAYLY R. C., DAGLEY S., GIBSON D. T. The metabolism of cresols by species of *Pseudomonas*. Biochem. J. **101**, 293, **1966**.
20. HORVATH R. S. Co-metabolism of methyl- and chloro-substituted catechols by an *Achromobacter* sp. possessing a new *meta*-cleaving oxygenase. Biochem. J. **119**, 871, **1970**.
21. HEISS G., MULLER C., ALTENBUCHNER J., STOLZ A. Analysis of a new dimeric extradiol dioxygenase from a naphthalenesulfonate-degrading sphingomonad. Microbiology **143**, 1691, **1997**.
22. SPAIN J. C., GIBSON D. T. Pathway for biodegradation of p-nitrophenol in a *Moraxella* sp. Appl. Environ. Microbiol. **57**, 812, **1991**.
23. KOLVENBACH B. A., LENZ M., BENDORF D., RAPP E., FOUSEK J., VLCEK C., SCHAFFER A., GABRIEL F. L. P., KOHLER H.-P. E., CORVINI P. F. X. Purification and characterization of hydroquinone dioxygenase from *Sphingomonas* sp. strain TTNP3. AMB Express **1**, 1, **2011**.
24. MATEO C., PALOMO J. M., FERNANDEZ-LORENTE G., GUISAN J. M., FERNENDEZ-LAFUENTE R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb. Technol. **40**, 1451, **2007**.
25. WALLIS M. G., CHAPMAN S. K. Isolation and partial characterization of an extradiol non- haem iron dioxygenase which preferentially cleaves 3-methylcatechol. Biochem. J. **266**, 605, **1990**.

26. HOLLENDER J., HOPP J., DOTT W. Cooxidation of chloro- and methylphenols by *Alcaligenes xylosoxidans* JH1. World J. Microbiol. Biotechnol. **16**, 445, **2000**.
27. HO K. L., CHEN Y. Y., LEE D. J. Functional consortia for cresol- degrading activated sludge: toxicity-to-extinction approach. Bioresource Technol. **101**, 9000, **2010**.
28. KARA F., DEMIREL G., TÜMTÜRK H. Immobilization of urease by using chitosan-alginate and poly(acrylamide-co-acrylic acid)/κ-carrageenan supports. Bioprocess Biosyst. Eng. **29**, 207, **2006**.
29. DHULSTER P., BARBOTIN J. N., THOMAS D. Culture and bioconversion use of plasmid-harboring strain of immobilized *E. coli*. Appl. Microbiol. Biotechnol. **20**, 87, **1984**.
30. JUNCA H., PLUMEIER I., HECHT H. J., PIEPER D. H. Difference in kinetic behavior of catechol 2,3-dioxygenase variants from a polluted environment. Microbiology **150**, 4181, **2004**.
31. TAKEO M., NISHIMURA M., SHIRAI M., TAKAHASHI H., KITAMURA C. H., NEGORO S. Purification and characterization of catechol 2,3-dioxygenase from aniline degradation pathway of *Acinetobacter* sp. YAA and its mutant enzyme, which resists substrate inhibition. J. Bioscen. Bioeng. **71**, 1668, **2007**.
32. KITA A., KITA S., FUJISAWA I., INAKA K., ISHIDA T., HORIIKE K., NOZAKI M., MIKI K. An archetypical extradiol-cleaving catecholic dioxygenase: the crystal structure of catechol 2,3-dioxygenase (metapyrocatechase) from *Pseudomonas putida* mt-2. Structure **7**, 25, **1999**.
33. HUANG Q., SHINDO H. Effects of cooper on the activity and kinetics of free and immobilized acid phosphatase. Soil Biol. Biochem. **32**, 1885, **2000**.