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Research Paper

Properties of catechol 1,2-dioxygenase in the cell free extract and immobilized extract of *Mycobacterium fortuitum*

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Abstract

Polycyclic aromatic hydrocarbons (PAH) are carcinogenic compounds which contaminate water and soil, and the enzymes can be used for bioremediation of these environments. This study aimed to evaluate some environmental conditions that affect the production and activity of the catechol 1,2-dioxygenase (C12O) by *Mycobacterium fortuitum* in the cell free and immobilized extract in sodium alginate. The bacterium was grown in mineral medium and LB broth containing 250 mg L^{-1} of anthracene (PAH). The optimum conditions of pH (4.0-9.0), temperature (5-70 °C), reaction time (10-90 min) and the effect of ions in the enzyme activity were determined. The *Mycobacterium* cultivated in LB shown higher growth and the C12O activity was two-fold higher to that in the mineral medium. To both extracts the highest enzyme activity was at pH 8.0, however, the immobilized extract promoted the increase in the C12O activity in a pH range between 4.0 and 8.5. The immobilized extract increased the enzymatic activity time and showed the highest C12O activity at 45 °C, 20 °C higher than the greatest temperature in the cell free extract. The enzyme activity in both extracts was stimulated by Fe^{3+}, Hg^{2+} and Mn^{2+} and inhibited by NH^{4+} and Cu^{2+}, but the immobilization protected the enzyme against the deleterious effects of K^{+} and Mg^{2+} in tested concentrations. The catechol 1,2-dioxygenase of *Mycobacterium fortuitum* in the immobilized extract has greater stability to the variations of pH, temperature and reaction time, and show higher activity in presence of ions, comparing to the cell free extract.

Key words: anthracene, enzyme activity, enzyme immobilization, biodegradation, waste treatment.

Introduction

In the current years, a high number of polluting compounds have been released into the environment by several anthropogenic activities. In particular, aromatic pollutants have caused the contamination of soil and water with negative impacts on environmental quality and healthy. Furthermore, it is already known that many aromatic compounds show high toxicity and cancerous proprieties for human and animals (Constantini *et al.*, 2009).

Over the last decades, it is increasing the interest in biological methodologies, collectively indicated as bioremediation that may help to reduce the risk of organic pollutants and effectively remediate polluted sites (Andreoni and Gianfreda, 2007). Liquid effluents contaminated with aromatic compounds can be efficiently processed with microbial enzymes, which compared to microorganisms have the advantage to not be affected by inhibitors of microbial growth; they act in various environmental conditions and are not susceptible to microbial competition (Mateo *et al.*, 2007).

The enzymes catechol dioxygenases add two oxygen atoms to the aromatic ring, disrupting chemical bonds and allowing opening this ring (Whiteley and Lee, 2006). The

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catechol 1,2-dioxygenase (C12O) (EC 1.13.11.1) contains Fe^{+3} as prosthetic group and belongs to the enzymes that make cleavage of catechol as intradiol (or ortho cleavage), producing *cis-cis* muconic acid (Tsai and Li, 2007). The use of catechol dioxygenases for bioremediation has been relatively little explored, although, there is a great potential to use these enzymes mainly associated with the use to bioreactors, to clean high amounts of wastewater contaminated with phenol, benzoate, fluorocatecol, bromocatecol, cholorocathecol, methilcathecol, herbicides (diuron), polychlorinated biphenyls, chloroethanes and others (Duran and Esposito, 2000; Macleod and Daugulis, 2005; Shunkova *et al.*, 2009). For using the catechol dioxygenase on a commercial scale, it is necessary to have large production in laboratory, and to do that, it is necessary to know the environmental conditions that can interfere in its activity.

Despite the many advantages using enzymes in the bioremediation of wastewater, it has been observed that free enzymes tend to show low stability under certain environmental conditions; so, the immobilization technology has been widely used, improving the activity, stability, specificity, selectivity and decreased inhibition (Fernandez-Lafuente *et al.*, 2000; Iyer and Ananthanarayar, 2008). Some studies have shown many supports that can be successfully used to enzymes immobilization (Mateo *et al.*, 2007), but using in large-scale, none of them have many qualities such as calcium alginate, which it is an easy, fast, nontoxic, inexpensive, robust, versatile and widely used to enzymes immobilization (Kalogeris *et al.*, 2006).

The majority of C12O enzymes were studied with gram negative bacteria and a small number of studies were with gram positive bacteria, in particular with actinobacteria (Shumkova *et al.*, 2009). The latest studies, it was isolated and characterized a powerful microbial consortium that can degrade aliphatic, mono- and poliaromatic hydrocarbons in culture medium and soil (Jacques *et al.*, 2007, 2008). The *Mycobacterium fortuitum*, a member of this consortium showed high degradation rates and high metabolic versatility. Although, in this study, it was evaluated some environmental conditions that affect the production and activity of the catechol 1,2-dioxygenase from *Mycobacterium fortuitum*, in the cell free extract and immobilized extract, to further bioremediation use in wastewater treatment.

Materials and Methods

Microorganism

An aromatic hydrocarbon degrading microbial consortium was obtained from the enrichment culture of a petrochemical landfarm site (Jacques *et al.*, 2007). This consortium is composed by five bacteria (*Mycobacterium fortuitum, Bacillus cereus*, *Microbacterium sp.*, *Gordonia polyisoprenivorans*, *Microbacteriaceae bacterium*, *Naphthalene-utilizing bacterium*) and a fungus, identified as *Fusarium oxysporum*. To this study, it was used the isolate *Mycobacterium fortuitum*, a gram-positive bacilli that has capacity to growth using one of the following compounds as unique carbon and energy source when incubated in mineral medium: anthracene, pyrene, phenanthrene, naphthalene, catechol, gentisic acid, toluene, 1-decene, 1-octene, ethanol and gasoline (Jacques *et al.*, 2007). The isolate was stored at 4 °C on nutritive agar medium (3 g of meat extract, 5 g of peptone, 15 g of agar in 1 L of distilled water, pH 7.0) with 250 mg L^{-1} of anthracene, a polycyclic aromatic hydrocarbon (PAH) (Kiyohara *et al.*, 1982).

Media and growth conditions

The isolate was inoculated in Erlenmeyer flasks with Tanner mineral medium (TMM) or Luria Bertani broth (LB), both containing 250 mg L^{-1} of anthracene as fine crystals (Merck™, Darmstadt, Germany). The TMM medium was composed by 0.04 g L^{-1} $CaCl_2$ $2H_2O$; 0.1 g L^{-1} KH_2PO_4; 0.8 g L^{-1} NaCl; 1.0 g L^{-1} NH_4Cl; 0.2 g L^{-1} $MgSO_4$ $7H_2O$; 0.1 g L^{-1} KCl. The micronutrients were prepared as follows: 0.1 mg L^{-1} $CoCl_2$ $6H_2O$; 0.425 mg L^{-1} $MnCl_2$ $4H_2O$; 0.05 mg L^{-1} $ZnCl_2$; 0.015 mg L^{-1} $CuSO_4$ $5H_2O$; 0.01 mg L^{-1} $NiCl_2$ $6H_2O$; 0.01 mg L^{-1} Na_2MoO_4 $2H_2O$; 0.01 mg L^{-1} Na_2SeO_4 $2H_2O$. All solutions were diluted in deionized water. The LB broth medium was composed by 5.0 g L^{-1} of meat extract; 10.0 g L^{-1} of tryptone; 10.0 g L^{-1} of NaCl. The pH was adjusted to 7.0 by adding aliquots of either HCl or NaOH. The media was sterilized by autoclaving at 121 °C for 20 min. Flasks were incubated at 30 °C with orbital shaking (150 rpm) with three replicates. After growth for 5 days in TMM, or 12 h in LB, an aliquot of 1.0 mL was taken from the cultures and diluted in saline solution, plated on agar nutritive medium and incubated for 24 h at 30 °C in the dark. Colonies were directly counted and expressed as cfu mL^{-1}. In these intervals, the C12O activity was assayed as described below.

Preparation of cell free extract

The cells were harvested from LB or TMM by centrifugation (10,000 rpm, for 10 min at 5 °C). The pellet was washed twice with phosphate buffer (50 mM, pH 7.0) and re-suspended in 10 mL of same buffer. Suspensions were sonicated with repeated 40 s bursts alternated with 1 min cooling in ice. Cells debris was centrifuged at 12,000 rpm for 15 min. Supernatants were used to enzyme assay and protein estimation.

Enzyme immobilization

The enzyme was immobilized with calcium alginate matrix technique (Kalogeris *et al.*, 2006). One milliliter of crude extract (9000 U) was suspended in 9 mL of 2% (w/v) sodium alginate prepared in 50 mM Tris-HCl buffer solu-

tion (pH 8.0). After homogenization of the mixture, the enzyme was dropped into 100 mL of 0.2 M $CaCl_2$ solution, using a pipette. Upon contact with the solution, the drops were gelled to form defined-sized spheres (3 mm of diameter) which remained in the solution under gentle agitation to complete gel formation. After 1 h of incubation, the beads were removed, washed twice with sterile distilled water and stored at 4 °C.

Enzyme assay and protein concentration

Activity of the soluble and immobilized catechol 1,2-dioxygenase enzyme was assayed spectrophotometrically by measurement the increase in absorbance at $\lambda = 260$ nm, corresponding to the formation of *cis,cis*-muconic acid. All determinations were made in duplicate for each sample. The reaction mixtures contained 1 mL of soluble or immobilized enzyme preparations; 1 mL of 0.8 mM catechol; 0.8 mL of 50 mM Tris-HCl buffer solution (pH 8.0) and 0.2 mL of 0.1 mM 2-mercaptoetanol (Hegman, 1966). After the addition of the enzyme (in both cell free extract and immobilized form), mixtures were incubated at 30 °C in a water-bath. At certain time intervals, aliquots were used to monitor the reaction progress in spectrophotometer. The protein concentration in supernatant cell free extract was estimated with method described by Bradford (1976), using bovine serum albumin as a standard.

Effect of pH, temperature and ions and maintenance of enzyme activity

The isolate was inoculated in Erlenmeyer flasks with LB broth containing 250 mg L^{-1} of anthracene as fine crystals and incubated at 30 °C, 150 rpm, with two replicates. After growth of 36 hours, the C12O activity was assayed in the cell free extract and immobilized extract as described above. The optimum pH was determined by measuring the activity at 30 °C over the pH range from 4.0 to 9.0, using the following buffers: 50 mM acetate (pH 4.0-5.5), 50 mM phosphate (pH 6.0-8.0) and 50 mM Tris-HCl (pH 7.0-9.0). The optimum temperature was determined by assaying the enzyme activity at various temperatures (from 5 to 50 °C) in 50 mM Tris-HCl buffer solution (pH 8.0). The ions tested were Cu^{2+}, Mg^{2+}, Hg^{2+}, Mn^{2+}, Fe^{2+}, K^{+} and NH_4^{+} at a concentration of 1.0 mM of each ion and 10 mM for NH_4^{+} in 50 mM Tris-HCl buffer solution (pH 8.0), at temperatures of 25 and 50 °C in cell free extract and immobilized extract respectively. Ions were not added to control than those in the buffer. The chemicals used were copper sulphate, magnesium chloride, mercury chloride, manganese chloride, iron chloride, potassium acetate and ammonium sulphate. The maintenance of activity of C12O was determined by measuring the activity in 50 mM Tris-HCl buffer solution (pH 8.0) during 90 min, at temperatures 25 and 50 °C in the cell free extract and immobilized extract respectively.

Results

Media and growth profile

The *Mycobacterium* growth slowly in the mineral medium and anthracene as only source of carbon and energy (Figure 1A). The growth rate was only 0.0024 h^{-1} and the maximum enzymatic activity (0.88 mM mL^{-1}) was occurred after 15 days. To reduce the incubation time, the *Mycobacterium* was cultivated in LB broth with anthracene, which increased the growth rate to 0.0138 h^{-1} and resulted after 36 h of incubation in high cells number and high enzymatic activity (1.62 mM mL^{-1}), being two-fold higher to that in the mineral medium (Figure 1B).

Effect of pH

To both cell free extract and immobilized extract the highest C12O enzyme activity was at pH 8.0 (Figure 2). In a range between pH 4.0 and 5.5, the enzyme activity was reduced, although, the immobilized extract showed two-fold higher than cell free extract, showing that the immobilization protected the enzyme against the deleterious effects of the low pH. In a range of pH from 6.5 to 7.5, the immobilized extract showed the activity 15% higher than cell free extract. This protection also was observed at pH 8.5, show-

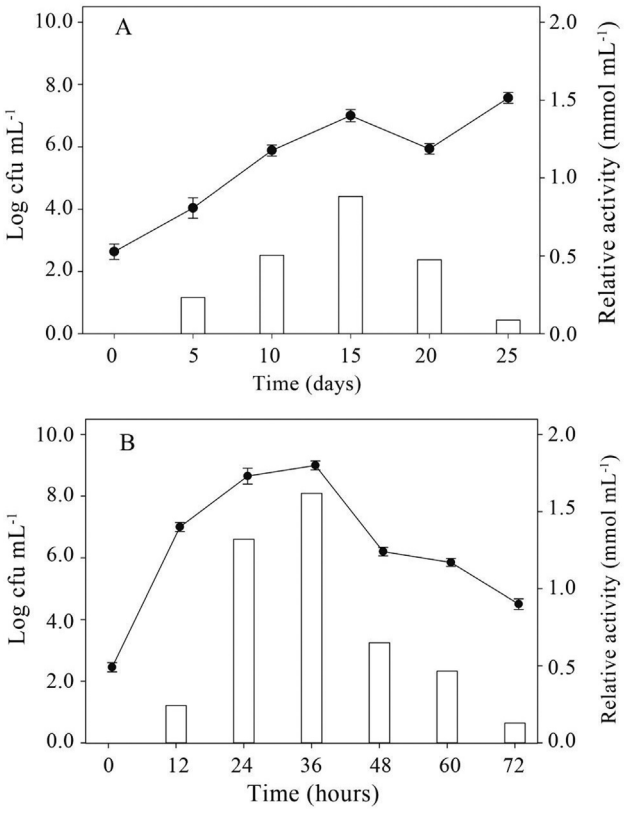

Figure 1 - Growth profile (lines) and enzyme activity of catechol 1,2-dioxygenase (bars) of isolate *Mycobacterium fortuitum* grown for 150 rpm at 30 °C in Tanner mineral medium (A) and in Luria-Bertani broth (B), both containing 250 mg L^{-1} of anthracene (data are mean of three replicates; error bars are standard error).

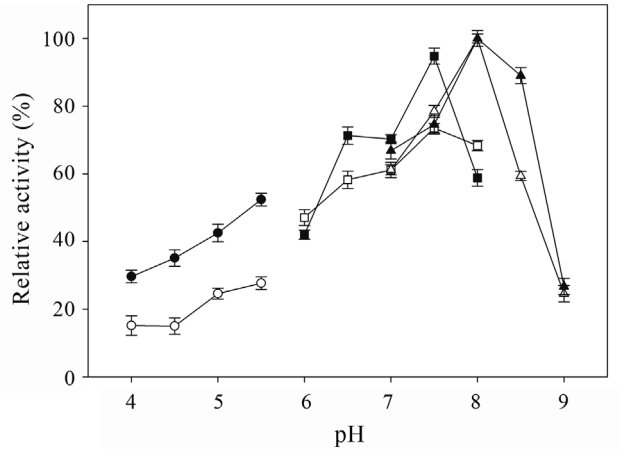

Figure 2 - Effect of pH in the catechol 1,2-dioxygenase activity present in the cell free extract (white symbols) and immobilized extract (black symbols) of *Mycobacterium fortuitum*. Buffers: acetate (pH 4.0 to 5.5 - cycle symbols), phosphate (pH 6.0 to 8.0 - square symbols), and tris-HCl (pH 7.0 to 9.0 - triangle symbols) (data are mean of two replicates; error bars are standard error).

ing that this effect is not just restrict to acid pH but also in alkaline pH. However, the activity was substantial reduced at pH 9.0 in the cell free extract as much as in the immobilized extract.

Effect of temperature

The cell free extract showed the C12O enzyme relative activity of 16% when incubated at 5 °C (Figure 3). This activity slightly increased until 20 °C, than strongly increased until the maximum activity at 25 °C. Above 35 °C, it was drastically reduced the activity achieving values near to zero at temperature 45 °C. Thus, the enzyme activity of the cell free extract was kept above 50% only in a temperature range between 20 and 35 °C. On the other hand, the immobilized extract showed zero enzymatic activity in the temperature range between 5 and 20 °C, than strongly increased until maximum activity at 45 °C, 20 °C higher than the greatest temperature in the cell free extract. The immobilized extract remained with enzyme activity (above 50%) in a high range of temperature between 35 and 70 °C. However, the extract immobilization promoted a high increase in enzyme activity at temperatures above 35 °C, but reduced the enzyme activity in temperatures under this value.

Maintenance of activity

The enzyme in the cell free extract showed activity during 70 min (Figure 4). In the first 20 min the C12O enzyme kept high activity, and then it decreased linearly until 70 min. The C12O enzyme activity in the immobilized extract was detected with more than 90 min, although, it showed a strongly decrease in the enzyme activity in the initial period when compared with cell free extract. Between 20 and 60 min, the activity was near to 50%, than, it decreased until 90 min of incubation. The enzyme immobilization increased the enzymatic activity time, where in the last 50 min of incubation, the enzyme activity of the immobilized extract showed 24% higher than the cell free extract activity, although, in the first 40 min free extract showed enzymatic activity 24% higher than the immobilized extract.

Effect of the ions

The cell free extract by *M. fortuitum* showed C12O activity inhibition by ions presence in the following order: $NH^{4+} > Cu^{2+} > K^{+} > Mg^{2+}$ (Table 1). However, the Mn^{2+}, Fe^{3+} and Hg^{2+} stimulated the enzyme activity, showing a relative increasing of more than 50%. In the immobilized extract, the C12O enzyme was inhibited only by the copper (11%) and ammonium (40%). The potassium did not affect the reaction; however, the magnesium inhibited the enzyme activity in the cell free extract, and in the immobilized ex-

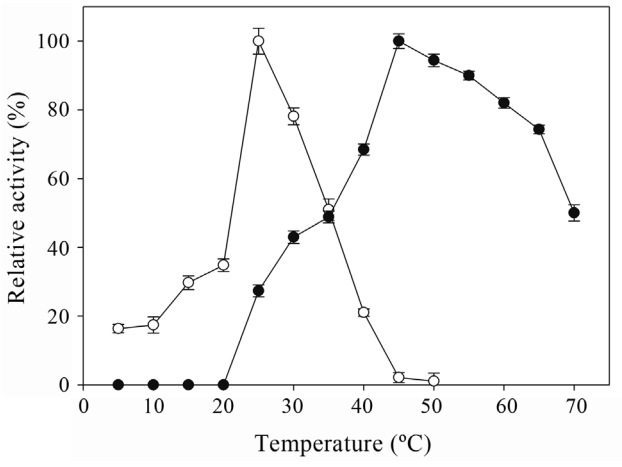

Figure 3 - Temperature effect in activity of the catechol 1,2-dioxygenase enzyme in the cell free extract (o) and immobilized extract (•) of *Mycobacterium fortuitum* (data are mean of two replicates; error bars represent standard error).

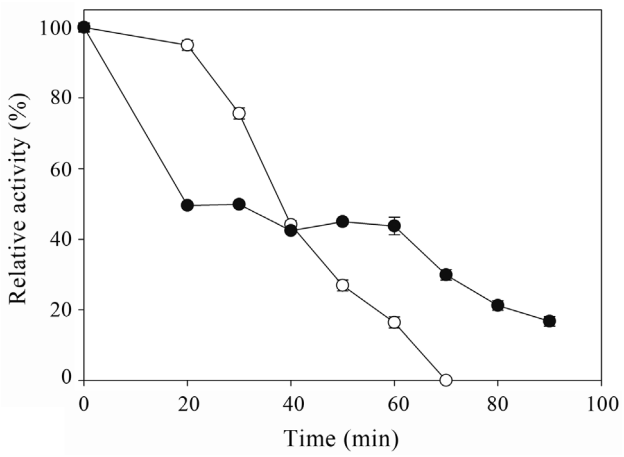

Figure 4 - During time of activity of the catechol 1,2-dioxygenase enzyme in the cell free extract (o) and immobilized extract (•) of *Mycobacterium fortuitum* (data are mean of two replicates; error bars are standard error).

Table 1 - Effect of ions in relative enzymatic activity of catechol 1,2-dioxygenase present in the cell free extract and immobilized extract of the *Mycobacterium fortuitum* (the control treatment is the activity in absence of ions).

Ion	Concentration (mM)	Relative activity (%)	
		Cell free extract	Immobilized extract
Control	-	100 ± 4.5*	100 ± 1.3
Fe^{3+}	1.0	156 ± 1.6	206 ± 0.7
Hg^{2+}	1.0	156 ± 2.1	161 ± 1.4
Mn^{2+}	1.0	151 ± 4.0	148 ± 0.4
Mg^{2+}	1.0	91 ± 3.4	138 ± 0.1
K^{+}	1.0	62 ± 1.4	102 ± 0.6
Cu^{2+}	1.0	60 ± 1.1	89 ± 2.5
NH_4^{+}	10.0	52 ± 2.5	60 ± 1.5

*Values are means ± standard error of the mean. (data are average of two replicates ± standard error).

tract was stimulated by this ion with 38% more activity. Fe^{3+}, Hg^{2+} and Mn^{2+} stimulated the enzyme activity of the both cell free and immobilized extract with highlighted efficiency, once, the Fe^{3+} ion showed activity twice higher than others in the immobilized extract.

Discussion

The low growth of the *Mycobacterium* isolate and the low C12O enzyme activity in the Tanner mineral medium is probably due to carbon limitation to the bacteria. The anthracene has very low solubility in water (0.076 mg L^{-1}), moreover, the carbon transferring rate from the solid phase (anthracene crystals) to the liquid phase (mineral medium) is very low (Johnsen *et al.*, 2005; Wich *et al.*, 2001), which it can limit the *Mycobacterium* growth, and consequently the C12O enzyme activity. However, the source of soluble carbon used in the medium together with the aromatic compounds seems that it not affected the C12O enzyme activity. This observation has important biotechnological implications, because it indicates that it is possible to obtain large amounts of cells of *Mycobacterium fortuitum* and with high activity of C12O from a non expensive culture medium and in a short period of time, since relatively small amounts of aromatic compound were added to the medium.

In a previews study, it was verified that the microbial consortium with the isolates *Mycobacterium fortuitum*, *Bacillus cereus*, *Microbacterium* sp., *Gordonia polyisoprenivorans*, they have been degraded 48%, 67%, and 22% of the anthracene, phenanthrene, and pyrene in the mineral medium, respectively, after 30 days of incubation in the soil (Jacques *et al.*, 2007). In our study, only the isolate *Mycobacterium fortuitum* exhibited the pick of the enzyme activity after 15 days of incubation with 0.9 mmol L^{-1} of anthracene. It shows the potential use of this kind of biotechnology.

The behavior of the C12O produced by *Mycobacterium fortuitum* in a pH range of 4.0 to 9.0 was similar to that showed by *Acinotobacter* (Briganti *et al.*, 1997) and *Rhodococcus* (Shunkova *et al.*, 2009), which all isolates showed high activity in the pH range between 6.0 and 8.0. The C12O enzyme immobilization with calcium alginate did not modified the optimum pH of enzyme activity (pH 8.0), result also showed by Kalogeris *et al.* (2006) which immobilized the C12O produced by *Pseudomonas putida* with calcium alginate. However, the enzyme immobilization promoted the increase in the activity in a pH range between 4.0 and 8.5, showing that the immobilization with the calcium alginate promoted an microenvironment where the enzyme has a higher activity, because it is partially protected from deleterious effect of the H^{+} and OH^{-} concentration (Matto and Husain, 2009; Quiroga *et al.*, 2011).

The enzyme activity might change with different temperatures and some metals soluble in the medium (Andreazza *et al.*, 2011). The variation in the C12O activity when changed the temperature occurred in this study was similar to that showed by Kalogeris *et al.* (19), which the difference of the optimal temperature for C12O activity produced by *P. putida* between the cell free extract and immobilized extract was 15 °C. The contrasting behavior of C12O activity in different temperature ranges in cell free and immobilized extract indicates that according to the temperature of the wastewater, there are advantages to use cell free or immobilized extract in the bioremediation treatment.

The immobilization can increase molecular stability of the immobilized enzyme, as a result of reduced thermal inactivation (Constantini *et al.*, 2009; Mateo *et al.*, 2000). Gottschalk and Jaenicke (1991) showed that immobilized enzymes have an increase of the enzyme rigidity, which it is commonly reflected by increasing the stability toward denaturation. The C12O activity showed higher duration in the immobilized extract than the cell free extract. A common mechanism of a multimeric enzyme (such as most catechol dioxygenases) inactivation is the dissociation of enzyme subunits (Kalogeris *et al.*, 2006). Immobilization of biocatalysts by entrapment in calcium alginate gel beads has been reported to improve significantly enzyme stability (Roy and Gupta, 2004). Under these circumstances, stabilization of the quaternary structure of a protein through immobilization could promote enzyme stability and improve its activity (Iyer and Ananthanarayan, 2008).

In many proteins, metal ions interactions play catalytic roles; in others, the metals appear to have a purely structural role. The Fe^{3+} was the ion with more stimulation in the activity of the C12O enzyme in the cell free extract and immobilized extract. The Fe^{3+} has a catalytic function and also a structural function to enzymes that can cleavage catechol intradiol, which the metal ion withdrawal has influence on the secondary structure of C12O, in particular a reduction of α-helices content (Di Nardo *et al.*, 2004). In relation to the

effect of the Mn in the intradiol-cleaving enzymes, Wang *et al.* (2006) showed that the activity of the C12O produced by *P. aeruginosa* was inhibited (only 32% of the relative activity, with 0.5 mM) by the presence of this ion in the mix reaction. It was not related that Mn^{2+} promoted the activity of the C12O, although, many enzymes have this ion in their structure and have their catalytic activity depending of Mn^{+2} (Crowley, 2000). Other wise, the same author have been find 18% of increase in the relative activity with Fe^{+2} ions, and 4% with Mg^{+2} (Wang *et al.*, 2006).

Hg^{2+} was the second ion that promoted the C12O activity. Some researchers showed that Hg^{2+} ion decreased the C12O enzyme activity (Matsumura *et al.*, 2004; Murakami *et al.*, 1998), and in some studies achieved almost 0% of the relative activity in the concentration of the 0.1 mM of Hg^{+2} (Murakami *et al.*, 1998). In the current study, this ion showed stimulation; however, the stimulation effect by Hg^{2+} ions was observed in other enzymes (Anthony, 1997; Karamitsu, 1968). Cu^{2+} drastically inhibited the C12O enzyme activity produced by *P. putida* (Wang *et al.*, 2006), *Geobacillus* sp. (Giedraityte and Kalëdienë, 2009) and *Alcaligenes xylosoxidans* (Yeom and Yoo, 1999). However, the inhibition was lower than that showed by these authors, mainly by the immobilized enzyme. This behavior was observed not only to Cu^{2+}, but also to all ions that showed inhibitory effect in the C12O activity (Mg^{2+}, K^{+}, Cu^{2+} and NH^{4+}). Krajewska (2004) showed that the ions diffusion through the solid barrier constituted by the substrate is low, reducing the inhibitors interference in the immobilized enzyme.

The catechol dioxygenases has a fundamental pathway in the carbon biochemical cycle and a high biotechnological potential in the treatment of liquid wastes contaminated with aromatics compounds. Nevertheless, there are few studies with catechol 1,2-dioxygenase from gram-positives and actinobacteria. In this study, it was demonstrated that the immobilization of the C12O enzyme from *Mycobacterium fortuitum* was significantly increased in the catalytic activity in the low and high pH conditions, high temperatures and in presence of the activity inhibitors. Extreme conditions such as above cited can be in the contaminated wastes, showing that the immobilization with a cheap substrate, not toxic and simple manipulation such as calcium alginate can contribute to turn this enzyme an efficient biotechnological alternative to treatment of liquid wastes contaminated with aromatic compounds.

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References

Andreazza R, Okeke Benedict C, Pieniz P, Brandelli A, Lambais MR, Camargo FAO (2011) Bioreduction of Cu(II) by cell-free copper reductase from a copper resistant *Pseudomonas* sp. NA. Biol Trace Elem Res 143:1182-1192.

Andreoni V, Gianfreda L (2007) Bioremediation and monitoring of aromatic-polluted habitats. App Microb Biotechnol 76:287-308.

Anthony R (1997) Ashton and Gabriele M. Siegel. Stimulation of spinach (*Spinacia oleracea*) chloroplast fructose-1,6-bisphosphatase by mercuric ions. FEBS Lett 408:30-32.

Bradford MM (1976) A rapid e sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Briganti F, Pessione E, Giunta C, Scozzafava A (1997) Purification, biochemical properties and substrate specificity of a catechol 1,2-dioxygenase from a phenol degrading *Acinetobacter radioresistens*. FEBS Lett 416:61-64.

Costantini AS, Gorini G, Consonni D, Miligi L, Giovannetti L, Quinn M (2009) Exposure to benzene and risk of breast cancer among shoe factory workers in Italy. Tumori 95:8-12.

Crowley JD, Traynor DA, Weatherburn DC (2000) Enzymes and proteins containing manganese: An overview. Met Ions Biol Syst 37:209-278.

Di Nardo G, Tilli S, Pessione E, Cavaletto M, Giunta C, Briganti F (2004) Structural roles of the active site iron(III) ions in catechol 1,2-dioxygenases and differential secondary structure changes in isoenzymes A and B from *Acinetobacter radioresistens* S13. Arch Biochem Bioph 431:79-87.

Durán N, Esposito E (2000) Potencial applicantions of oxidative enzyme and phenoloxidase-like compounds in wastewater and soil treatment: A review. App Catalysis B 28:83-99.

Fernandez-Lafuente R, Guisan, J.M.; Ali, S.; Cowan, D. (2000) Immobilization of functionally unstable catechol-2,3-dioxygenase greatly improves operational stability. Enzyme Microb Tech 26:568-573.

Giedraityte G, Kalëdienë L (2009) Catechol 1,2-dioxygenase from α-naphthol degrading thermophilic *Geobacillus* sp. strain: Purification and properties. Cent Eur J Biol 4:68-73.

Gottschalk N, Jaenicke R (1991) Authenticity and Reconstitution of Immobilized Enzymes: Characterization and Denaturation/Renaturation of Glucoamylase II. Biotechnol Appl Bioc 14:324-335.

Hegman GD (1966) Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. J Bacteriol. 91:1140-1154.

Iyer PV, Ananthanarayan L (2008) Enzyme stability and stabilization - Aqueous and non-aqueous environment. Process Biochem 43:1019-1032.

Jacques RJS, Okeke BC, Bento FM, Peralba MCR, Camargo FAO (2007) Characterization of a polycyclic aromatic hydrocarbon-degrading microbial consortium from a petrochemical sludge landfarming site. Bioremed J 11:1-11.

Jacques RJS, Okeke BC, Bento FM, Teixeira AS, Peralba MC, Camargo FAO (2008) Microbial consortium bioaugmentation of a polycyclic aromatic hydrocarbons contaminated soil. Bioresource Technol 99:2637-2643.

Johnsen AR, Wick LY, Harms H (2005) Principles of microbial PAH-degradation in soil. Environ Pollut 133:71-84.

Macleod CT, Daugulis AJ (2005) Interfacial effects in a two-phase partitioning bioreactor: Gedradation ps polycyclic aromatic hydrocarbons (PAHs) by a hydrophobic *Mycobacterium*. Process Biochem 40:1799-1805.

Kalogeris E, Sanakis Y, Mamma D, Christakopoulos P, Kekos D, Stamatis H (2006) Properties of catechol 1,2-dioxygenase from *Pseudomonas putida* immobilized in calcium alginate hydrogels. Enzyme Microb Tech 39:1113-1121.

Karamitsu HK (1968) Mercury(II) stimulation of malate dehydrogenase activity. The J Biol Chem 243:1016-1021.

Kiyohara H, Nagao K, Yana K (1982) Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. Appl Environ Microbiol 43:454-457.

Krajewska B (2004) Application of chitin and chitosan based materials for enzyme immobilizations: A review. Enzyme Microb Tech 35:126-139.

Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R (2007) Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb Tech 40:1451-1463.

Mateo C, Abian O, Fernandez-Lafuente R, Guisan JM (2000) Increase in conformational stability of enzymes immobilized on epoxy-activated supports by favoring additional multipoint covalent attachment. Enzyme Microb Tech 26:509-515.

Matsumura E, Ooi S, Murakami S, Takenaka S, Aoki K (2004) Constitutive synthesis, purification, and characterization of catechol 1,2-dioxygenase from the aniline-assimilating bacterium *Rhodococcus* sp. AN-22. J Biosci Bioeng 98:71-76.

Matto M, Husain Q (2009) Calcium alginate-starch hybrid support for both surface immobilization and entrapment of bitter gourd (*Momordica charantia*) peroxidase. J Mol Catal. B-Enzym 57:164-170.

Murakami S, Wang CL, Naito A, Shinke R, Aoki K (1998) PuriWcation and characterization of four catechol 1,2-dioxygenase isozymes from the benzamide-assimilating bacterium *Arthrobacter* species BA-5-17. Microbiol Res 153:163-171.

Quiroga E, Illane S CO, Ochoa NA. Barberis, S. (2011). Performance improvement of *araujiain*, a cystein phytoprotease, by immobilization within calcium alginate beads. Process Biochem. 46:1029-1034.

Roy I, Gupta NM (2004) Hydrolysis of starch by a mixture of glucoamylase and pullulanase entrapped individually in calcium alginate beads. Enzyme Microb Technol 34:26-32.

Shumkova ES, Solyanikova IP, Plotnikova EG, Golovleva LA (2009) Phenol degradation by *Rhodococcus opacus* Strain 1G. Appl Biochem Microbiol 45:43-49.

Tsai SC, Li YK (2007) Purification and characterization of a catochel 1,2-dioxygenase from a phenol degrading *Candida albicans* TL3. Arch Microbiol 187:199-206.

Wang CL, You SL, Wang SL (2006) Purification and characterization of a novel catechol 1,2-dioxygenase from *Pseudomonas aeruginosa* with benzoic acid as a carbon source. Process Biochem 41:1594-1601.

Wick LY, Colangelo T, Harms H (2001) Kinetics of mass transfer-limited bacterial growth on solid PAHs. Environ Sci Tech 35:354-361.

Whiteley CG, Lee JD (2006) Enzyme technology and biological remediation. Enzyme Microb Tech 38:291-316.

Yeom SH, Yoo YJ (1999) Removal of benzene in a hybrid bioreactor. Process Biochem 34:281-88.