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The effects of trace elements, cations, and environmental conditions on protocatechuate 3,4-dioxygenase activity

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ABSTRACT: Phenanthracene is a highly toxic organic compound capable of contaminating water and soils, and biodegradation is an important tool for remediating polluted environments. This study aimed to evaluate the effects of trace elements, cations, and environmental conditions on the activity of the protocatechol 3,4-dioxygenase (P3,4O) enzyme produced by the isolate *Leifsonia* sp. in cell-free and immobilized extracts. The isolate was grown in Luria Bertani broth medium (LB) amended with 250 mg L^{-1} of phenanthrene. Various levels of pH (4.0-9.0), temperature (5-80 °C), time (0-90 min), trace elements (Cu^{2+}, Hg^{2+} and Fe^{3+}), and cations (Mg^{2+}, Mn^{2+}, K^{+} and NH_4^{+}) were tested to determine which conditions optimized enzyme activity. In general, the immobilized extract exhibited higher enzyme activity than the cell-free extract in the presence of trace elements and cations. Adding iron yielded the highest relative activity for both cell-free and immobilized extracts, with values of 16 and 99 %, respectively. Copper also increased enzyme activity for both cell-free and immobilized extracts, with values of 8 and 44 %, respectively. Enzyme activity in the phosphate buffer was high across a wide range of pH, reaching 80 % in the pH range between 6.5 and 8.0. The optimum temperatures for enzyme activity differed for cell-free and immobilized extracts, with maximum enzyme activity observed at 35 ºC for the cell-free extract and at 55 ºC for the immobilized extract. The cell-free extract of the P3,4O enzyme exhibited high activity only during the first 3 min of incubation, when it showed 50 % relative activity, and dropped to 0 % after 60 min of incubation. By contrast, activity in the immobilized extract was maintained during 90 min of incubation. This isolate has important characteristics for phenanthrene biodegradation, producing high quantities of the P3,4O enzyme that forms part of the most important pathway for PAH biodegradation.
Keywords: enzyme activity, enzyme immobilization, biodegradation, waste treatment

Introduction

Polycyclic aromatic hydrocarbons (PAH) are highly toxic compounds that pose risks to humans and animals. Factors such as trace elements and environmental conditions interfere with microorganisms' metabolisms for PAH biodegradation. Studies on biodegradation have revealed its significant promise for reducing pollution and cleaning up polluted sites (Tao et al., 2007; Chang et al., 2011; Janbandhu and Fulekar, 2011). Bioremediation of PAH-contaminated areas using biodegradation techniques may be an efficient alternative for removing these contaminants from the environment.

Enzymes used for degradation can be divided into two groups: peripheral and fission enzymes. Peripheral enzymes are responsible for recognizing and converting the PAHs in degradable molecules, and fission enzymes pass these molecules through the common pathways for energy generation and carbon routing in the microbial cells (Mishra et al., 2001). The enzyme protocatechol 3,4-dioxygenase (P3,4O) (EC 1.13.11.3) cleaves the aromatic ring to form the intermediary compound protocatechol. The extradiol enzymes open the aromatic ring adjacent to the hydrocarboxilic carbons. However, the *meta* pathway that originates the 2-hydroximuconic semialdehyde will be transformed in pyruvate and acetaldehyde. In this pathway, catechol 2,3-dioxygenase belongs to the class of enzymes that cleave the aromatic ring to form the core intermediary catechol, and protocatechol 4,5-dioxygenase to the class that cleave the aromatic ring to form the compound protocatechol (Caldwell, 2000). However, enzyme activity and its applicability for biodegradation require more study.

Protocatechol 3,4-dioxygenase belongs to the class of enzymes that cleave the intradiol group. It contains the Fe (III) as a prosthetic group and many subunits α and β form different quaternary structures $(\alpha\beta)_n$, where n is a number varying between 3 and 12 (Harayama et al., 1992). Some microorganisms can produce P3,4O for the biodegradation of organic compounds, including *Acinetobacter lwoffii* (Kahng et al., 2002), *Acinetobacter calcoaceticus* (Zaborsky and Schwartz, 1974), and *Pseudomonas aeruginosa* (Ohlendorf et al., 1987). However, more studies are needed to understand the behavior this enzyme with other isolates and under other conditions.

The use of enzymes can be an efficient alternative for remediation of environments contaminated with PAHs. Enzymes can be used to treat contaminated environments in different ways. Furthermore, enzymes have many advantages over conventional treatments such as chemical complexation (Durán and Esposito, 2000). Enzyme immobilization promotes beneficial effects for cat-

alytic efficiency as well as for kinetics and stability properties (Degaldillo and Rodriguez-Nogales, 2005). It also offers some advantages for bioremediation (Fernandez-Lafuente et al., 2000). However, trace elements, cation ions, and environmental conditions can interfere with the activity of the P3,4O enzyme. The aim of this study was to evaluate the effect of trace elements, cations, and environment conditions on cell-free and immobilized P3,4O enzyme activity.

Materials and Methods

Microorganism, media, and growth conditions - An aromatic hydrocarbon degrading microbial consortium was obtained from the enrichment culture of a petrochemical landfarm (Jacques et al., 2007). From this consortium, the 16S rRNA region of Isolate 6 was sequenced. The sequence obtained was compared with the most similar sequences retrieved from GenBank and the results did not show an exact classification for this bacterium (Jacques et al., 2007; 2009). Thus, Isolate 6 was characterized as a member of the genus *Leifsonia* with 98 % similarity (Figure 1).

The *Leifsonia* sp. was inoculated in Erlenmeyer flasks with Luria Bertani broth (LB) amended with 250 mg L^{-1} of phenanthrene as fine crystals (Merck-™, Darmstadt, Germany). The LB broth medium was composed of 5.0 g L^{-1} of meat extract; 10.0 g L^{-1} of tryptone; and 10.0 g L^{-1} of NaCl. All solutions were diluted in deionized water. The pH was adjusted to 7.0 by adding aliquots of either HCl or NaOH. The media were 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, cells were harvested by centrifuging (10,000 rpm) for 15 min at 4 °C.

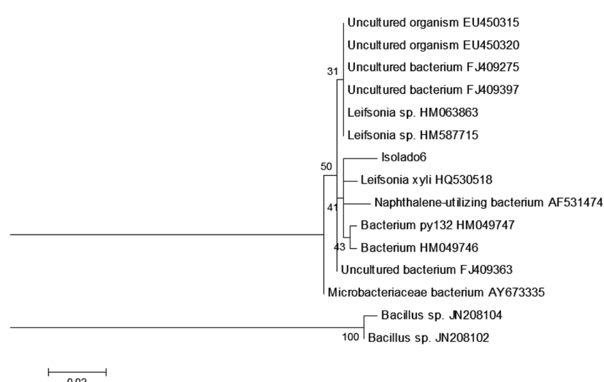

Figure 1 – Phylogenetic tree showing the affiliations of the isolate obtained in this study and isolates retrieved from GenBank. The numbers at branch points indicate bootstrap values based on 1000 replicates. Molecular phylogenetic analysis was carried out with the Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model.

Enzyme immobilization - The enzyme was immobilized using the sodium alginate matrix technique (Giedraityte and Kalediene, 2009). 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 solution (pH 8.0). After homogenization of the mixture, the enzyme was added with a dropper to 100 mL of solution of 0.2 M $CaCl_2$. Upon contact with the solution, the drops gelled to form regularly-sized spheres (3 mm diameter), and these remained in the solution under gentle agitation to complete gel formation. After 1 h incubation, the beads were removed, washed twice with sterile distilled water and stored at 4 °C.

Enzyme activity - The enzyme activity of the both cell-free and immobilized P3,4O enzyme was assayed spectrophotometrically (absorbance at $\lambda = 290$ nm), corresponding to the formation of cis-muconic acid. The reaction mixtures contained 1 mL of soluble or immobilized enzyme preparations; 1 mL of 0.12 mM protocatechol acid; and 1.0 mL of the 50 mM Tris-HCl buffer solution (pH 7.5), as described by Fujisawa and Hayaishi (1968). All determinations were made in duplicate for each sample. After the addition of the enzymes to the cell-free and immobilized extracts, mixtures were incubated in water baths at 35 and 55 °C, respectively. At regular time intervals thereafter, aliquots were used to monitor the reaction progress in the spectrophotometer.

Effects of trace elements and cations - The trace elements tested were Cu^{2+}, Hg^{2+}, and Fe^{2+}. The cations tested were NH_4^+, Mg^{2+}, Mn^{2+}, and K^+. All trace elements and cations were tested at concentrations of 1.0 mM, with the exception of NH_4^+, which was tested at 10 mM. Each cation and metal was dissolved in 50 mM Tris-HCl buffer solution (pH 8.0) at 25 °C in the cell-free extract. The chemicals used were copper sulfate, mercury chloride, iron chloride, magnesium chloride, manganese chloride, potassium acetate, and ammonium sulfate.

Effects of pH, temperature, and time - Optimum pH was determined by measuring activity at 30 °C over the pH range of 4.0 to 9.0, using the following buffers: 50 mM acetate (pH 4.0, 4.5, 5.0, and 5.5), 50 mM phosphate (pH 6.0, 6.5, 7.0, 7.5, and 8.0), and 50 mM Tris-HCl (pH 7.0, 7.5, 8.0, 8.5, and 9.0). Optimum temperature was determined by assaying enzyme activity at various temperatures (from 5 to 50 °C). Temporal variation in enzyme activity was determined by measuring enzyme activity during 90 min, at 10-min intervals. In the temperature and temporal tests Tris-HCl buffer (50 mM, pH 8.0) was used because both cell-free extract and immobilized enzyme had the highest activity at the alkaline medium in these soils.

Effects of trace elements and cations - Different trace elements and cations exhibited different effects on the enzyme activity of the P3,4O (Figure 2). In general, the

immobilized extract exhibited higher activity than the cell-free extract with the presence of the trace elements and cations, except in the case of Mn^{2+}. Enzyme activity of the P3,4O in the cell-free extract was highly inhibited in the presence of K^+, NH^{4+}, and Mg^{2+}, with relative activity declining by 83, 69 and 66 %, respectively. The inhibition of the immobilized extract was low for K^+ and Mg^{2+}, with relative activity values of 14 and 36 %, respectively. The presence of ammonium with the P3,4O exhibited the same level of inhibition for both cell-free and immobilized extract of the enzyme activity.

Among the trace elements, iron showed the highest relative activity for both cell-free and immobilized extract with values of 16 and 99 %, respectively (Figure 2). Copper also increased enzyme activity for both cell-free and immobilized extract with values of 8 and 44 %, respectively. Although Hg inhibited enzyme activity of the cell-free extract, it greatly increased activity of the immobilized extract, by 40 %. Immobilization protected the enzyme from the deleterious effects of K^+ and Hg^{+2} and also increased enzyme activity in the presence of Fe^{+3} and Cu^{+2}.

Effects of pH - Activity of the P3,4O enzyme produced by the isolate *Leifsonia* sp. peaked at pH 8.0 in the Tris-HCl buffer with almost 100 % of relative activity (Figure 3). The phosphate buffer exhibited high enzyme activity, reaching 80 % in the pH range between 6.5 and 8.0. The activity of the P3,4O in acetate buffer was up to 40 % in the pH range between 4.0 and 5.5, showing that this enzyme is very active over a wide range of pH values.

Enzyme activity of the P3,4O was high at pH 7.5 in the phosphate buffer for both cell-free and immobilized extract (Figure 3). In general, immobilization of the P3,4O enzyme with sodium alginate yielded an increase in stability, increasing the pH range with high enzyme activity. The immobilized extract showed high enzyme activities in the pH range between 4.0 and 6.5 (between 40 and 75 % of relative activity), and in the pH range between 8.0 and 9.0 (relative activity above 80 %). The immobilization of the P3,4O enzyme exhibited the best results of enzyme activity in the different pH ranges.

Effects of temperature - The cell-free and immobilized extracts of P3,4O enzyme exhibited different behaviors (Figure 4). Enzyme activity of the P3,4O for the cell-free extract was high until 40 °C and decreased substantially above that temperature. By contrast, enzyme activity of the immobilized extract was high only for tempera-

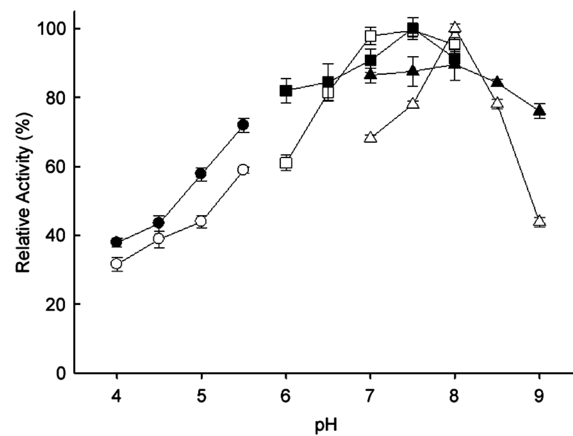

Figure 3 – Effects of variation in pH on protocatechol 3,4-dioxigenase activity in the cell-free extract (white symbols) and the immobilized extract (black symbols) of a *Leifsonia* sp. Buffers: acetate (from pH 4.0 to 5.5 - circle symbols), phosphate (from pH 6.0 to 8.0 - square symbols), and tris-HCl (from pH 7.0 to 9.0 - triangle symbols). Error bars are standard errors of the means.

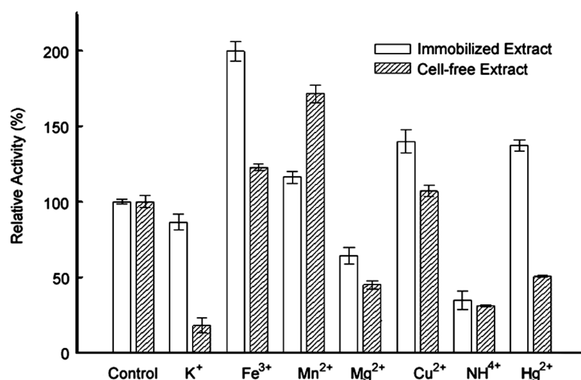

Figure 2 – Effects of trace elements and cations on the relative enzymatic activity of the protocatechol 3,4-dioxygenase (P3,4O) produced by *Leifsonia* sp. in the cell-free and immobilized extracts. Error bars represent standard errors. The control treatment was enzyme activity in the absence of the ions.

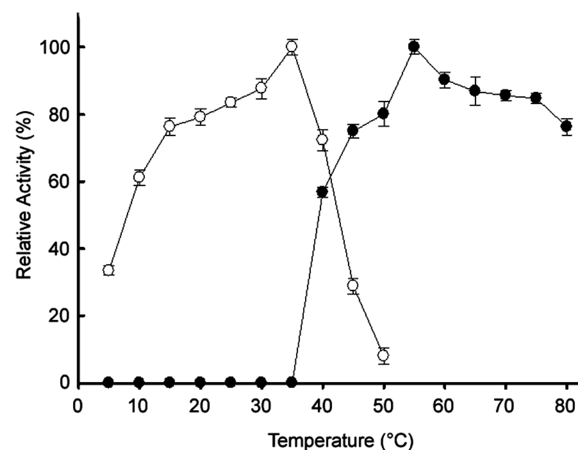

Figure 4 – Effects of different temperatures of the cell-free (white symbols) and immobilized (black symbols) extracts on the relative activity of the protocatechol 3,4-dioxygenase produced by *Leifsonia* sp., after 30 min of incubation. Error bars are the standard errors of the means.

tures greater than 40 °C. The optimum temperature of the enzyme activity was different for the cell-free and immobilized extracts, with maximum enzyme activities observed at 35 °C and 55 °C, respectively. In summary, the cell-free extract exhibited high enzyme activity (up to 60 %) in the temperature range between 10 and 40 °C, while immobilization of the enzyme increased the optimum pH range with high enzyme activity in the temperature range between 45 and 80 °C, with activity values around 80 % of relative activity.

Effects of time - Temporal variation of P3,4O enzyme activity for both cell-free and immobilized extracts showed the same pattern, decreasing over time (Figure 5). A relative activity of 100 % was only achieved at the starting time. The cell-free extract of the P3,4O enzyme exhibited high activity only for the first 30 min of incubation, during which it showed 50 % of relative activity, and achieved values close to 0 % activity after 50 min of incubation. The immobilized enzyme maintained a relative activity of more than 50 % for the first 45 min. Furthermore, immobilization of the P3,4O maintained enzyme activity during the first 90 min of incubation. For all time periods, enzyme activity of the immobilized extract was higher than that of the cell-free extract (Figure 5), indicating that immobilization could be the best way to use the P3,4O enzyme.

Discussion

PAHs are recalcitrant compounds with high toxicity and carcinogenic properties for humans and animals (Costantini et al., 2009). PAH pollution requires efficient treatments for cleaning up the environment and reducing the risks these compounds pose for living

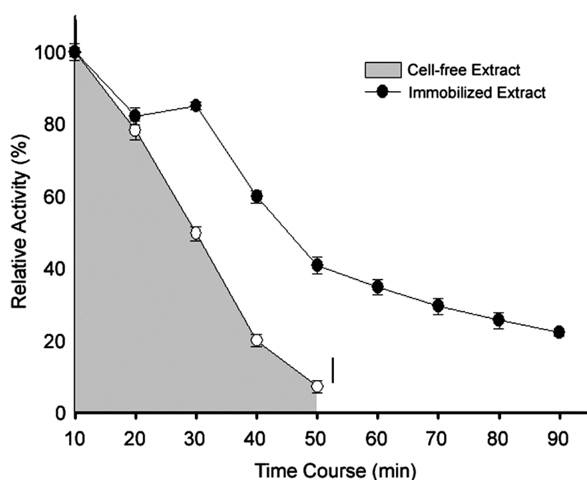

Figure 5 – Variation over time of the relative activity of the protocatechol 3,4-dioxygenase (P3,4O) in the cell-free extract and immobilized extract produced by a Leifsonia sp. Error bars are the standard errors of the means.

organisms. One such treatment is high-efficiency biodegradation using enzymes produced by microorganisms (Chang et al., 2011; Janbandhu and Fulekar, 2011). The enzyme protocatechol 3,4-dioxygenase (P3,4O) plays an important role in some pathways for biodegrading PAHs, including phenanthrene. However, trace elements, cations, and environmental conditions are important influences on enzyme activity and consequently for biodegradation.

Cations and heavy metals were added to the reaction mixture as sulfate, acetate, and chloride, all inexpensive compounds. These products can thus be easily used in bioreactors, if bioremediation technology is adopted at a large scale by industry. Fe^{3+} increased P3,4O enzyme activity up to 71 %. Iron is an important trace element for many cellular functions, where it is intrinsically linked with the metabolism of the PAHs, participating in the structure of the dioxygenases enzymes. Some authors have evaluated the structure of and the cleavage of the aromatic ring by intradiol enzymes such as P3,4O (Bertini et al., 1995). Through these studies, it was possible to observe that the presence of the Fe^{3+} increased the activity of these enzymes for degradation by the cleavage orientation in the aromatic ring and because this trace element is part of the molecular structure of these enzymes as well as cofactor in the prosthetic group (Harayama et al., 1992). This could help explain the effect of iron in increasing P3,4O enzyme activity.

The strong inhibition caused by ammonium sulfate, even with the immobilized extract, is related to the fact that ammonium sulfate can destructure the conformation of the alginate due the presence of dissolved ions (Ishibashi et al., 1990). Other negative effect of the ammonium sulfate on cell-free extract can be promoted by the denaturation of the proteins breaking the hydrogen bonds and disulfide. This inhibitory effect was illustrated with *Pseudomonas putida* by Ishibashi et al. (1990).

Protocatechol 3,4-dioxygenase activity was high at pH 7.0 in the phosphate buffer, and at pH 8.0 in the Tris-HCl buffer. Maximum activity was thus influenced both by variation in pH and by the change of buffer. Bull and Ballou (1981) and Buchan et al. (2000) reported the greatest activity for this enzyme at pH 8.0 in phosphate buffer, and in the pH range between 8.0 and 9.0 in the Tris-HCl buffer. For this enzyme it is thus impossible to determine the optimum pH without looking at the buffer, since the buffer promotes activity, probably through the type and quantity of dissolved ions in the solution. However, the phosphate buffer promoted high activity across a wide pH range, and is thus more useful for bioremediation.

The cell-free extract of the P3,4O enzyme showed a high relative activity, close to 80 %, at 15 °C, and activity continued to increase up to 35 °C. This shows that this enzyme has a high biotechnological potential for use in the treatment of effluents contaminated with PAHs, due to the high activity in the temperature range between 15 and 40 °C for the cell-free extract. High tem-

peratures, up to 35 °C, decreased the cell-free extract enzyme activity. Optimum temperatures for dioxygenase enzyme activity can vary. This variation is due to the isolate that produces the enzyme for biodegradation, and the optimum temperature range has been reported to range between 25 and 85 °C, depending on the isolate species (Yang et al., 2008).

The use of enzymes is promising. Some practical problems remain with regard to the free forms, including the instability of the structure when it is removed from its natural environment and processed in non-optimum conditions, which promotes low stability and short enzyme activity times. With enzyme immobilization, the system stabilizes the enzyme structure, making it more resistant to environmental conditions. Immobilized enzymes are easily recovered and can be reused in enzymatic processes of continuous operation for a large variety of uses in different models of bioreactors (Krajewska, 2004). Immobilized enzymes can also be used in a wider range of environmental conditions than cell-free extracts, and thus appear to be the best choice for biodegradation under a variety of environmental conditions.

Conclusions

Fe, Mn, and Cu can improve P3,4O enzyme activity, and are important amendments for biodegradation. The immobilization of the crude extract can help protect the P3,4O enzyme and increase enzyme activity with cations (K^+), trace elements (Hg^{+2}), and environmental conditions such as pH, temperature, and time. The use of phosphate buffer promoted high enzyme activity for the P3,4O across a wide pH range. The isolate *Leifsonia* sp. showed important characteristics for biodegrading phenanthrene, producing high quantities of the P3,4O enzyme that forms part of the most important pathway for PAH biodegradation and which can be used in bioreactors for bioremediation.

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