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Production of a Recombinant Catechol 2,3-Dioxygenase for the Degradation of MicropollutantsDomenico Celesia^a, Isabelle Salzmänn^b, Emanuel Vaz Porto^b, Floriane Walter^b, Cindy Weber^b, Rémy Dufresne^b, and Simon Crelier^{a,b}

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Abstract: Phenolic compounds such as catechol represent a particular type of micropollutant whose high stability prevents rapid decay and metabolization in the environment. We successfully cloned a catechol 2,3-dioxygenase (C2,3O) from *Pseudomonas putida* mt-2 and expressed it in *Escherichia coli* BER2566. The biomass isolated from shake-flask fermentations was used to partially purify the enzyme. The enzyme proved unstable in clarified liquid fractions (50 mM Tris buffer, pH 7.6) and lost more than 90% of its activity over 7 h at 25 °C. In the presence of 10% acetone, the process was slowed down and 30% residual activity was still present after 7 h incubation. Storage of the enzyme in clear liquid fractions also proved difficult and total inactivation was achieved after 2 weeks even when kept frozen at –20 °C. Lowering the storage temperature to –80 °C preserved 30% activity over the same period. Only minor reactivation of the affected enzyme could be achieved after incubation at 20 °C in the presence of FeSO₄ and/or ascorbic acid. Activity loss seems to be due mostly to Fe²⁺ oxidation as well as to subunit dissociation in the tetrameric structure. However, complete degradation of 1.0 mM catechol could be achieved at 20 °C and pH 7.6 over a 3 h period when using a suspension of whole cells or alginate-encapsulated cells for the biotransformation. Contrary to the clear liquid fractions, these forms of biocatalyst showed no significant sign of inactivation under the working conditions.

Keywords: Bioconversion · Catechol · Catechol 2,3-dioxygenase · Enzyme · Micropollutants

1. Introduction

Micropollutants are compounds of various types such as heavy metals and organic molecules (e.g. textile dyes, UV-blocking agents, hormones, drug metabolites or pesticides) that have spread in the environment due to human activities and over the years they have become a global environmental threat. Despite their relatively low concentration levels in soil, sediment, water and air, micropollutants can be traced over the whole food chain and sometimes in the most remote locations. Their deleterious effects have already been measured in a variety of ecosystems and include acute toxicity, loss of fertility, birth defects, sex change, and endocrine disruption.^[1,2]

In its Directive 2008-105-EC, the European Commission has issued a list of molecules or groups of substances that should be kept under control or eliminated.^[3] This list features a large variety of organic molecules including biocides, pesticides, polyaromatic

hydrocarbons (PAH), polybrominated biphenylethers (PBDE) and alkylphenols (AP). Many of these substances belong to the sub-class of persistent organic pollutants (POPs), i.e. organic compounds that resist natural abatement through biological, photochemical or chemical processes. Because of their stability, POPs have the ability to bioaccumulate and potentially exert adverse impacts on human health and ecosystems.

Phenols represent a very large family of industrially relevant building blocks, intermediates and final products. They are used in various sectors such as oil extraction, mining, organic synthesis, polymer processing and paper industry. Every year approximately 10 million tons of phenolic compounds are produced and a great quantity is unduly released into the environment.^[4] A significant source of phenols and polyphenols is the paper industry, where the black liquor resulting from wood pulping contains a large amount of phenolic substances deriving from chemical modification (e.g. methylation, halogenation and polymerization) of lignin components. This further increases the toxicity of the material and its resistance to degradation.^[4] In spite of that, lignin waste is usually considered and treated as low-impact waste and often released in the environment without proper handling.^[5,6] Among lignin phenolic compounds, catechol and its halogenated derivatives have a particular relevance since they are present in some pesticides and also used sometimes as antioxidants for personal care products. For agencies such as the International Agency for Research on Cancer (IARC), the US Environmental Protection Agency (USEPA) and the Canadian Environmental Protection Agency (CEPA), catechols are registered as carcinogenic and considered more toxic than phenol itself (both *in vitro* and *in vivo*), which corresponds to Group 2B of IARC.

Catechol plays a key role in the aerobic metabolism of several bacteria, fungi and yeast and some of them were found able to grow in contaminated sites using organic pollutants as carbon source. For instance *Pseudomonas* sp., *Stenotrophomonas* sp., *Acinetobacter* sp., *Alcaligenes* sp., *Pleurotus* sp., *Trametes* sp. and *Aspergillus* sp. have enzymes capable of metabolizing catechol since it is formed as an intermediate in certain metabolic pathways, such as toluene and xylene degrading routes of *Pseudomonas putida*.^[7–11]

In comparison with chemical oxidation methods, enzymatic degradation has the advantage of being efficient at mild temperatures, pressures and pH values. There are large libraries of microorganisms from which enzymes can be selected according to their specific activity, selectivity and stability. The enzyme catechol 2,3-dioxygenase (C2,3O) [EC 1.13.11.2] has the ability to oxidize catechol as well as its halogenated derivatives^[12] by extradiol cleavage, a reaction shown in Scheme 1 that releases 2-hydroxymuconic acid semialdehyde (2-HMS).

The goal of the present study was to characterize the expression, stability and degrading capability of a recombinant catechol 2,3-dioxygenase towards catechol. To this effect we have cloned a C2,3O from *Pseudomonas putida* mt-2 and expressed it in *Escherichia coli* BER2566 under control of the *LacI* regulation. The recombinant enzyme was then produced by fermentation and used to degrade catechol in synthetic effluents, either in the form of a clarified, partially purified extract or using intact cells (free or encapsulated in an alginate matrix).

2.9 Degradation of Catechol

Catechol degradation was measured as already stated by monitoring the formation of 2-HMS (the reaction product) at 375 nm. The reaction was conducted at room temperature (20 °C) in 50 mL Falcon tubes and under three different sets of conditions: a) reaction mixture consisted in 3 mL of 10 mM catechol, 24 mL of 50 mM Tris-HCl with 150 mM NaCl (pH 7.6) and 3 mL of concentrated biomass;

b) reaction mixture consisted in 3 mL of 10 mM catechol, 21 mL of same Tris buffer as condition a) and 6 mL of alginate beads;

c) same conditions as in a), except that the biomass was substituted with 3 mL of crude lysate.

In all situations, the total volume of the reaction mixture was 30 mL. The tubes were placed on a rotary agitator set at 30 rpm. Samples were taken at specific time intervals and the course of the reaction was monitored until full completion or until equilibrium was reached.

3. Results and Discussion

3.1 Cultivation of Recombinant Cells

The different cultivation trials have shown that the Terrific Broth medium yielded the highest amount of active enzyme. Supplementing the medium with FeSO₄ upon induction with IPTG did not lead to any increase in enzyme production. On the other hand, it slightly increased the growth of *E. coli* cells and greatly enhanced the protein concentration and dry matter content of the suspension. Following biomass harvesting, cell disruption with the French press was found more effective than *via* the glass beads homogenizer. With LB broth medium, protein concentration in the lysate was 0.918 ± 0.014 mg/mL using the homogenizer, whereas significantly higher values were obtained with the French press device, as shown in Table 1.

Table 1. Optical density, dry matter content in the concentrated cell suspension prior to disruption, protein concentration in the lysate and C_{2,3O} specific activity in the clarified lysate for the different cultivation media. Data refer to disruption with the French press device.

Cultivation medium	Final OD ₆₀₀	Dry matter %	Protein conc. [mg/mL]	Spec. Activity [U/mg]
Lysogenic Broth (LB)	6.4	4.69	6.43	11.75
Terrific Broth (TB)	16.2	7.2	24.01	20.04
TB+FeSO ₄	16.6	10.04	35.54	16.38

3.2 Enzymatic Conversion of Catechol

Catechol degradation was tested in three different configurations, namely using free cells, encapsulated cells and a clarified cell lysate as biocatalyst (as described in section 2.9). The specific activity of catechol 2,3-dioxygenase in the lysate was measured prior to the bioconversion trials and reached 8.43 U/mg. All reactions were run in triplicate, and the corresponding results are shown in Fig. 1.

Analysis of the conversion curves in Fig. 2 shows that the free cell suspension is the most efficient of all three since it approaches complete conversion of catechol after about 90 min, despite a *ca.* 30 min lag phase. The conversion curve for encapsulated cells features a shorter lag phase of *ca.* 15 min before taking off. From

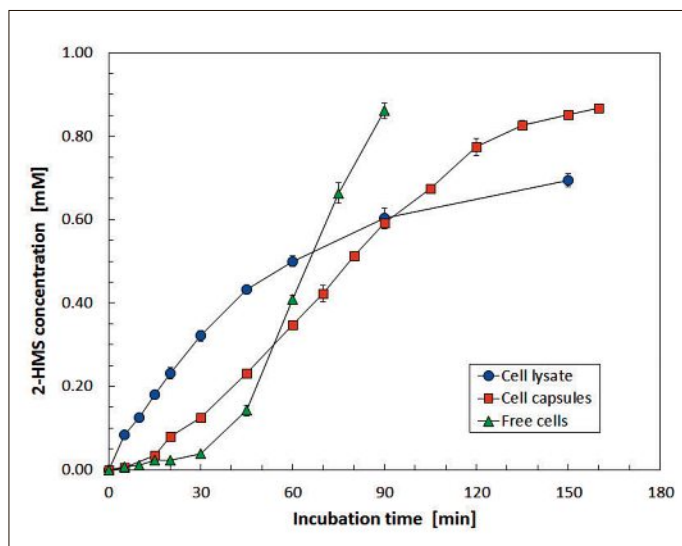


Fig. 1. Enzymatic conversion of catechol with free cells, alginate-encapsulated cells and cell lysate at room temperature (20 °C) and pH 7.6. Initial catechol concentration: 1.0 mM

this point product concentration steadily increases, although at a slower rate. This is most likely due to mass transfer limitations that affect the transport of substrate and product through the alginate matrix. Reaction with the clarified lysate appears to be fastest over the first 45 min, but then gradually slows down while the others accelerate. Furthermore, the concentration of 2-HMS seems to reach a plateau at *ca.* 80% conversion, which suggests a severe inactivation of catechol 2,3-dioxygenase under the above-described operating conditions.

3.3 Enzyme Stability

In the course of this study, loss of enzyme activity was observed on several other occasions. This phenomenon is described in the literature and is most probably due to the dissociation of subunits (most known catechol 2,3-dioxygenases are homotetramers), or to oxidation/altering binding of the Fe²⁺ atom located in the catalytic site.^[16,17] Consequently, a series of experiments was performed in order to quantify and better characterize the inactivation of the recombinant C_{2,3O} in a clarified cell lysate.

To this effect the loss of enzymatic activity was monitored under different sets of conditions. In a first set of experiments, a sample of clarified cell lysate was incubated for a few hours in 50 mM Tris buffer with 150 mM NaCl (pH 7.6) at 25 and 37 °C, and also at 25 °C in the presence of 10% acetone, which has been described as a protective agent against oxidation.^[18] The results of these experiments are shown in Fig. 2.

It can be seen that C_{2,3O} rapidly loses its activity under all conditions. More than 90% are lost over 7 h at 25 °C, but it seems the presence of 10% acetone effectively slows the process down. Increasing temperature to 37 °C only accelerates the inactivation process, and no activity can be detected after 5 h.

Retention of activity upon storage at various temperatures was also investigated. A sample of clarified cell lysate was stored in a 50 mM Tris buffer with 150 mM NaCl (pH 7.6) under a liquid form at 4 °C, and frozen at -20 and -80 °C. Samples were taken at selected time intervals and the remaining enzyme activity measured. Since the material was kept in separated tubes, each sample was thawed only once, thus eliminating a possible bias from multiple freezing/thawing steps. The results of these measurements are plotted in Fig. 3.

The loss of activity at 4 °C is rapid and clearly shows that the enzyme present in the cell lysates cannot be stored and is not suited for implementation at room temperature, as shown also

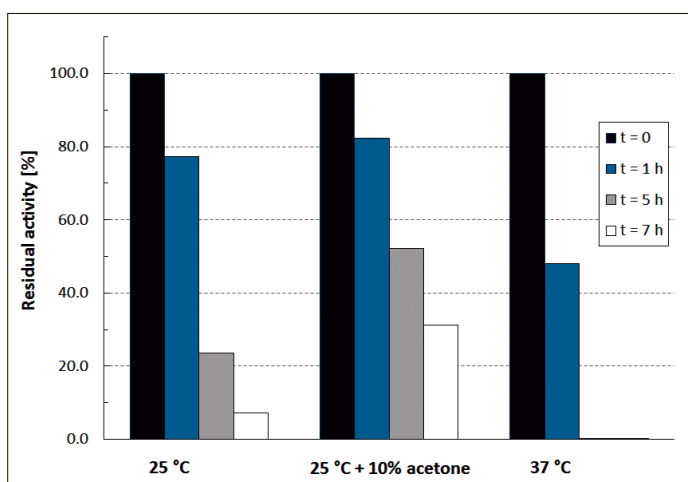


Fig. 2. Activity loss of catechol 2,3-dioxygenase in a clarified cell lysate over incubation time under different conditions. Residual activity was measured at room temperature.

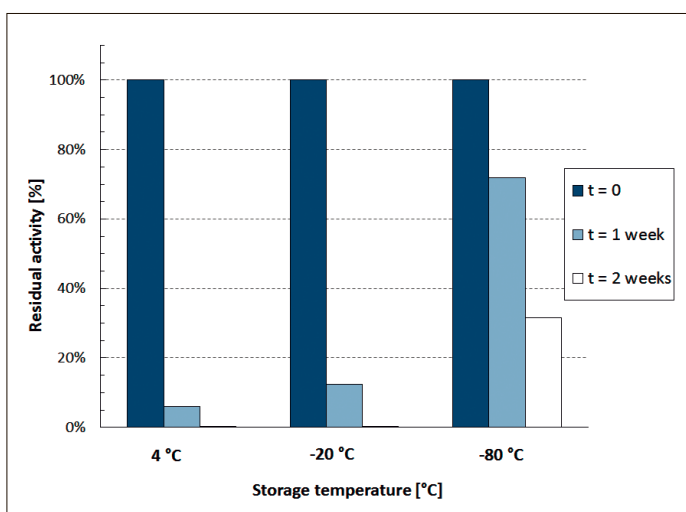


Fig. 3. Stability of catechol 2,3-dioxygenase in a clarified cell lysate when stored at different temperatures. Residual activity was measured at room temperature.

in the trials of Fig. 3. Freezing the material at $-20\text{ }^{\circ}\text{C}$ does not seem to dramatically extend the lifetime of the biocatalyst. It is only at $-80\text{ }^{\circ}\text{C}$ that the enzyme seems to retain about 30% of its initial activity after 2 weeks storage time. This is certainly not sufficient to guarantee an efficient use in the context of liquid effluent decontamination.

3.4 Activity Recovery Test

After a 45 min incubation under vacuum in the presence of FeSO_4 , ascorbic acid and ascorbic acid + FeSO_4 , the activity of catechol 2,3-dioxygenase was measured and compared to a control, untreated sample that had undergone activity loss at room temperature. The results are displayed in Figure 4.

In spite of some differences in the activity levels, it is very difficult to identify a clear cut, positive influence of either Fe^{2+} ions or ascorbic acid on the activity of the enzyme. The largest impact is observed with 2 mM ascorbic acid but the measurement could only be taken once and this result cannot be deemed conclusive. These results are only in partial agreement with previous observations by Cerdan^[16] or Nozaki.^[19] The enzymes were however not of the same origin and a more systematic investigation would be necessary to draw conclusions.

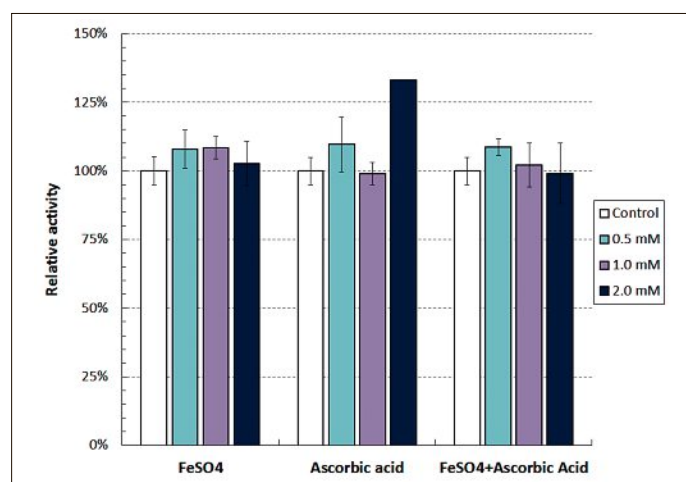


Fig. 4. Impact of FeSO_4 and ascorbic acid on the activity of catechol 2,3-dioxygenase after a 45 min incubation under vacuum. Activity was measured at room temperature.

4. Conclusion

Catechol 2,3-dioxygenase (C2,3-O) from *Pseudomonas putida* mt-2 has been successfully cloned and expressed in *Escherichia coli* BER2566. Shake flask fermentations at 500 mL scale were optimized and the collected biomass was used for direct biotransformations as well as for partial enzyme purification. French press gave the best results with respect to cell disruption, and the enzymatic activity of C2,3O has been characterized in the clarified lysate.

A 1.0 mM aqueous solution of catechol in a 50 mM Tris buffer of pH 7.6 was used as a synthetic effluent and the free cell suspension as well as alginate-encapsulated cells proved capable of converting catechol almost completely into 2-hydroxyomuconic acid semialdehyde (2-HMS).

The enzyme from the clear cell lysate, on the other hand, proved to lose activity rapidly under the tested experimental conditions. This observation was confirmed by a series of stability measurements which showed a rapid, irreversible loss of activity even in the presence of a stabilizing agent such as acetone. Contacting the inactivated enzyme with FeSO_4 and ascorbic acid showed none of the expected activity recovery.

Furthermore, it seems that a lysate is not suited for medium term storage and should be used fresh, since it otherwise loses its activity in less than two weeks when frozen at $-20\text{ }^{\circ}\text{C}$.

Catechol 2,3 dioxygenase nevertheless has potential for the degradation of catechol-like contaminants and encapsulation of the recombinant cells together with immobilization of the enzyme on a support seem to be the most promising approaches for future investigation and developments.

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