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Production of a Recombinant Catechol 2,3-Dioxygenase for the Degradation of Micropollutants

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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 $FeSO_4$ and/or ascorbic acid. Activity loss seems to be due mostly to Fe^{2+} 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]

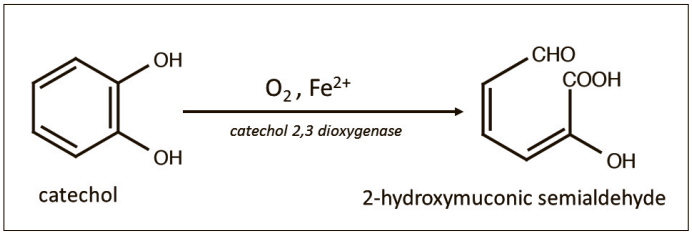
In its Directive 2008-105-EC, the European Commission has issued a list of molecules or groups of substances that should be kept under control or eliminated.[3] This list features a large variety of organic molecules including biocides, pesticides, polyaromatic hydrocarbons (PAH), polybrominated biphenylethers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



Scheme 1. Formation of 2-hydroxymuconic acid semialdehyde (2-HMS) through extradiol cleavage of catechol by catechol 2,3-dioxygenase (C2,3O).

2. Experimental

2.1 *Materials*

Tryptone, yeast extract and nutrient agar were supplied by Biolife Italiana (Milano, IT). Glycerol, K_2HPO_4 and KH_2PO_4, Trizma-base, Tris-HCl, catechol, low-viscosity sodium alginate, calcium chloride and L-ascorbic acid were purchased from Sigma-Aldrich (Buchs, CH). IPTG was supplied by BioVectra (Charlottetown, CA). $FeSO_4$ was purchased from Merck (Darmstadt, DE), and imidazole from Acros Organics (Geel, BE).

2.2 *Instrumentation*

All absorbance measurements were performed using a Libra S12 (Biochrom Ltd, Cambridge, UK) or an Agilent 8453/89090A UV/Vis spectrophotometer (Agilent, Santa Clara, USA). Small volumes were centrifuged with a MF 20-R equipment (Awel SAS, Blain, FR). Larger volumes were handled using a HiCen XL centrifuge equipped with the F8S-6x1000y rotor (Herolab GmbH, Wiesloch, DE). Cell disruption was achieved using a PreCellys 24 glass beads homogenizer (Bertin Instruments, Montigny-le-Bretonneux, FR) and a Stansted Fluid Power SPCH-10 French press homogenizer (Stansted Fluid Power Ltd, Harlow, UK). Chromatographic separations were performed with an Äkta Explorer 100 fitted with a 1.6 x 2.5 cm HisTrap FF 5mL column and with a Frac 950 fraction collector, (GE Healthcare, Little Chalfont, UK). A Nisco VARJ1 coaxial air flow nozzle (Nisco Engineering, Zürich, CH) was used for the production of alginate beads.

2.3 *Preliminary Isolation and Cloning of Catechol 2,3-dioxygenase Gene*

Catechol 2,3-dioxygenase (C2,3O) is encoded by the *xylE* gene on the TOL plasmid of *Pseudomonas putida* mt-2. For the isolation of the gene, restriction analysis, ligation, His-Tag addition and transformation of *E. coli* BER2566, established procedures were employed.[13] After selection of recombinant colonies, the success of cloning was confirmed by sequencing. A selected colony was then grown in Lysogenic (LB) broth and 700 µL aliquots were mixed with 300 µL glycerol in microtubes for subsequent storage at –80 °C. The same recombinant cells were also spread onto LB agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% nutrient agar, pH 7) containing 0.2 mM IPTG and 100 µg/mL ampicillin, and grown overnight at 37 °C. A 100 mM catechol solution was then sprayed on the colonies. The successful expression of the enzyme was confirmed by the development of a yellow color corresponding to the formation of 2-HMS.

2.4 *Scaling-up*

A colony was first inoculated in 50 mL LB broth spiked with 50 µL of ampicillin (100 µg/mL), and incubated overnight at 37 °C whilst being shaken at 180 rpm. This pre-inoculum was then split between two Erlenmeyer flasks containing 500 mL LB broth or Terrific Broth (12 g/L tryptone, 24 g/L yeast extract, 0.4% glycerol v/v, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4, pH 7.2) and 500 µL of ampicillin. The flasks were left to incubate under the same conditions as above and recombinant protein production was induced with 2 mL 100 mM IPTG when OD_{600} reached 0.4–0.6. In some instances, Terrific Broth growing flasks were also supplemented with $FeSO_4$ at a concentration of 5 mM. From the time of inoculation, growth was continued at 30 °C for 18 h.

2.5 *Harvesting and Analytics*

The cultures were then centrifuged at 7000 g for 45 min, and the pellet washed with 50 mM Tris-HCl buffer containing 150 mM NaCl, pH 7.6. After washing the suspension was centrifuged again, the supernatant discarded and the pellet re-suspended in a small amount of Tris buffer pH 7.6. The concentrated biomass suspension was then lysed either with a Precellys 24 glass beads homogenizer or by passing it twice through a French press at ambient temperature and 1000 bar pressure. The cell lysate was then clarified by centrifugation under the same conditions as above, and the supernatant was used for analysis and degradation trials. Protein concentration in the lysate was measured with the Bradford method using BSA as a standard.[14] Enzymatic activity was measured reading the increase of absorbance due to the 2-HMS formation at λ = 375 nm. At this wavelength the molar extinction coefficient for 2-HMS is 33400 $M^{-1}cm^{-1}$ at pH 7.6, according to ref. [15].

One unit of enzyme activity is defined as the amount of enzyme required to generate 1 µmol of product per minute at 25 °C. Assays were performed in 1.5 mL cuvettes containing 100 µL of 10 mM catechol solution, 20 to 100 µL of clarified lysate and completing to a final volume of 1 mL with 50 mM Tris-HCl buffer plus 150 mM NaCl, pH 7.6

2.6 *Stability of the Enzyme*

Catechol 2,3-dioxygenase is prone to self-oxidation of the Fe^{2+} nucleus as well as to dissociation of its tetrameric structure, which results in a strong loss of enzymatic activity.[16] The stability of the enzyme has thus been measured under various conditions.

2.7 *Reactivation of the Enzyme*

Based on a previous investigation of the mechanism of C2,3O inactivation,[16] we attempted to recover enzymatic activity using ascorbic acid as an antioxidant to regenerate the Fe^{2+} atom. To this effect a crude lysate sample of known activity was left to inactivate at room temperature (20 °C) for 4 h. This material was then distributed in 100 µL aliquots in 1 mL Eppendorf tubes and each of them was incubated for 45 min with ascorbic acid and/or $FeSO_4$ at known concentrations, the final volume in the tube being 1.0 mL. Incubation was performed in the absence of oxygen using a vacuum pump. C2,3O activity was then measured and compared with an untreated sample. Activity of the untreated control was equal to 3.46 U/mg.

2.8 *Gel Formation and Encapsulation Method*

The recombinant *E. coli* cells were encapsulated within calcium alginate gel using the following protocol: 5 mL of a concentrated cell suspension (OD_{600}=306) were gently mixed with an equal volume of a 3% (w/v) solution of sodium alginate in 50 mM Tris-HCl with 150 mM NaCl (pH 7.6). The resulting suspension was then extruded through a 500 µm diameter nozzle into a 3.0% $CaCl_2$ solution to form alginate microspheres. In order to obtain droplets with diameter <1 mm and regular size, air pressure was set to 1.2 bar, suspension flow rate set to 2.5 mL/min and dropping height fixed to 20 cm. The beads were gently stirred for 30 min in the gelling bath before they were removed using a strainer and subsequently used as immobilized biocatalyst for catechol degradation trials.

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 $FeSO_4$ 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 C2,3O 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 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.

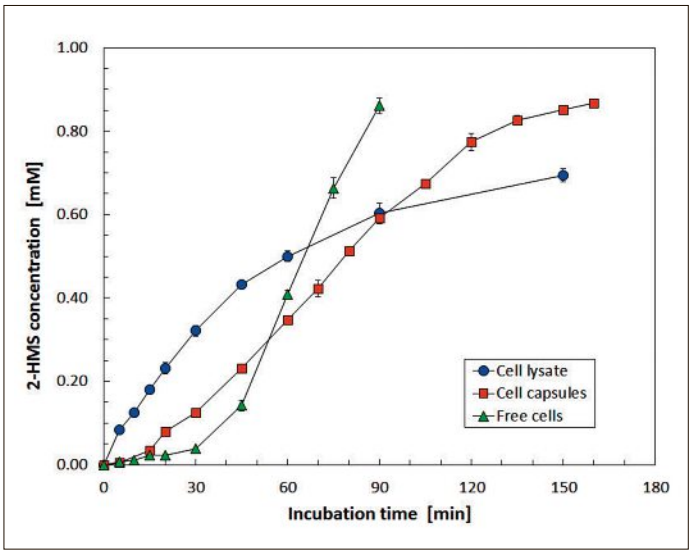


Fig. 1. Enzymatic conversion of catechol with free cells, alginate-encapsulated cells and cell lysate at room temperature (20 °C) and pH 7.6. Initial catechol concentration: 1.0 mM

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/altered binding of the Fe^{2+} 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 C2,3O 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 C2,3O 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

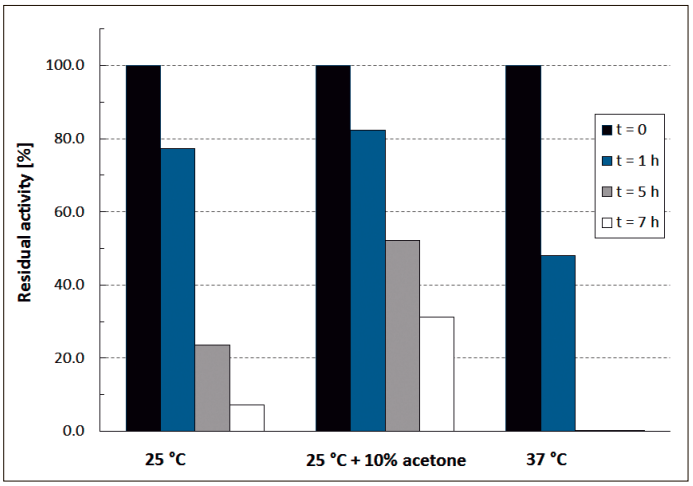


Fig. 2. Activity loss of catechol 2,3-dioxygenase in a clarified cell lysate over incubation time under different conditions. Residual activity was measured at room temperature.

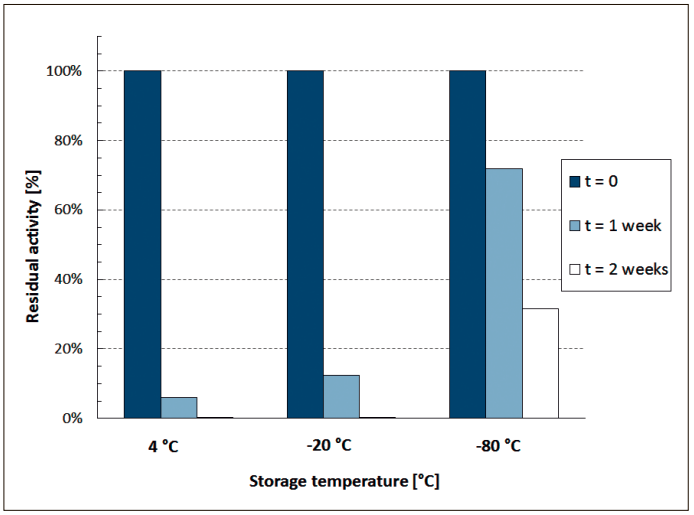


Fig. 3. Stability of catechol 2,3-dioxygenase in a clarified cell lysate when stored at different temperatures. Residual activity was measured at room temperature.

in the trials of Fig. 3. Freezing the material at –20 °C does not seem to dramatically extend the lifetime of the biocatalyst. It is only at –80 °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.

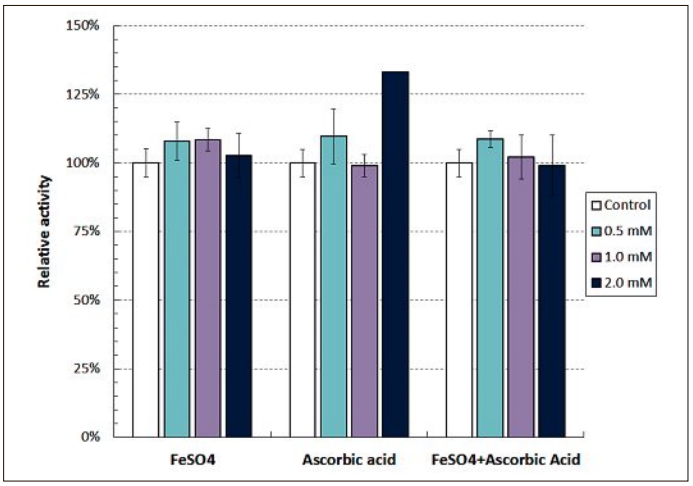


Fig. 4. Impact of $FeSO_4$ and ascorbic acid on the activity of catechol 2,3-dioxygenase after a 45 min incubation under vacuum. Activity was measured at room temperature.

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-hydroxymuconic 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 °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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