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ENZYMATIC NEUTRALIZATION OF ENDOCRINE DISRUPTING ACTIVITY OF POTENT COMPOUNDS

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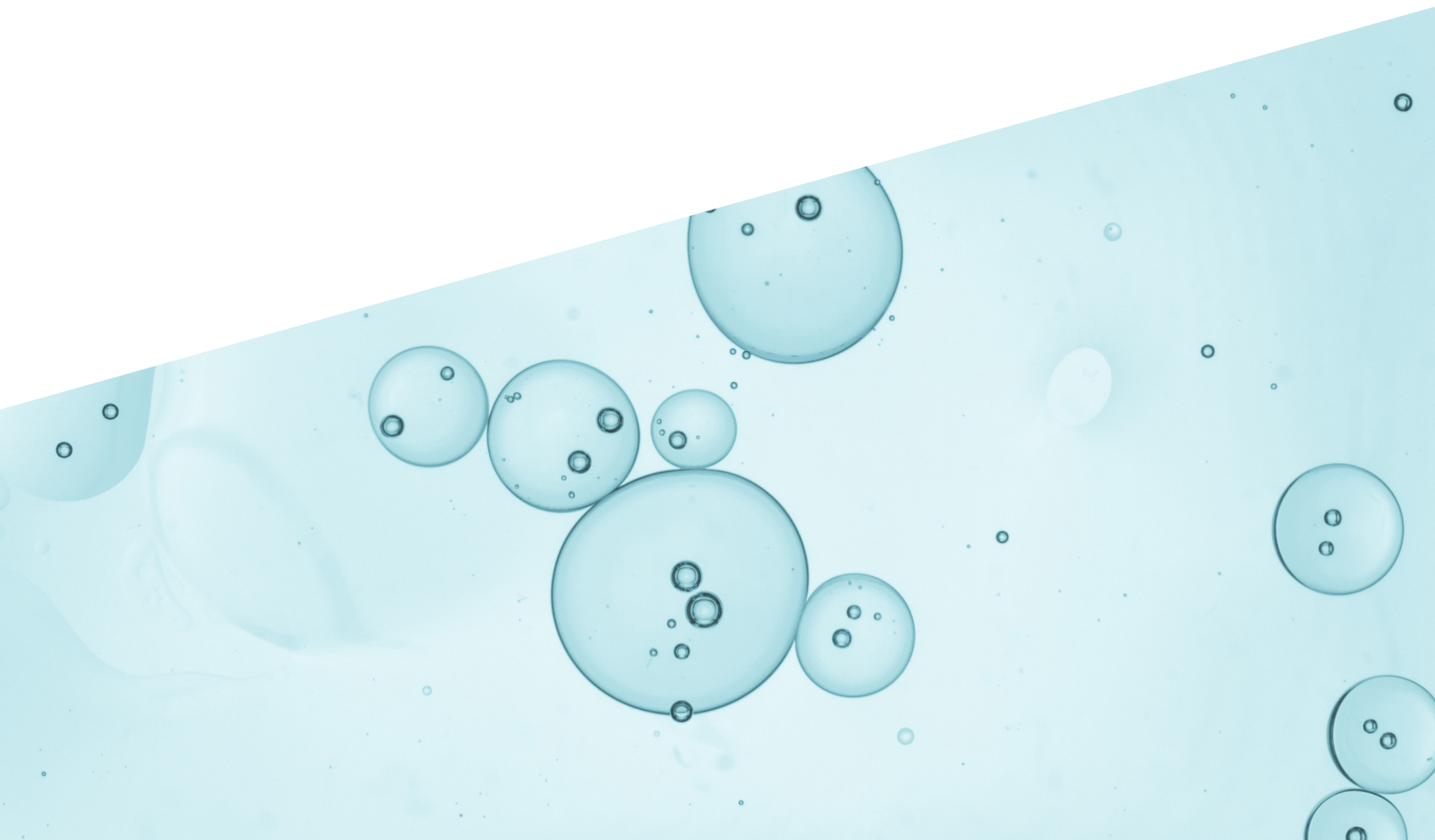

1-Introduction

1.1 Background

Endocrine disrupting compounds (EDCs) have been defined as exogenous substances that alter the functioning of the endocrine system of a living organism and consequently cause adverse health effects to that intact organism, or its progeny, or (sub)populations (WHO, 2002).

They include natural and synthetic estrogens, bisphenol A (BPA), polychlorinated biphenyls (PCB), dioxins, some pesticides (DDT), alkylphenols (nonylphenol), detergents, and plasticizers (phthalates), polybrominated flame retardants, phytoestrogens (coumestrol, genistein), mycotoxins (zearalenone) and some heavy metals (Mita et al., 2010; Harris et al., 1997, Tamagawa et al., 2006). However, the potencies of these compounds are widely varied: for example, potencies of phthalates are from 106 to 5.107 times less than that of 17ß-estradiol (E2) (Harris et al., 1997) and that of BPA is 104 less than that of E2 also (Suzuki et al., 2003). The list of EDCs resulting from human activities and found in wastewater is long. However, in general, natural hormones (estrone (E1), E2, estriol (E3)) and synthetic hormones (17ß-ethinylestradiol (EE2), mestranol) are the major contributors to the estrogenic activity observed in sewage effluents and the receiving water bodies (Auriol et al., 2006).

Drinking water is produced from surface or groundwater resources, which also serve as receiving water bodies for effluents from wastewater treatment plants (WWTP). This results in drinking water contamination by EDCs (Auriol et al., 2006). Beyond human health concerns, WWTP effluents can discharge contaminants into rivers at levels sufficient to induce vitellogenin biosynthesis and feminization in male fish (Tamagawa et al., 2006). Birds, reptiles and mammals in polluted areas undergo alterations of their endocrine reproductive system (Auriol et al., 2006).

To avoid human health and environmental consequences of EDCs, these compounds have to be removed from effluents so as to reach “no effect” concentrations. This is indeed a challenge since estrogenic compounds have adverse effects down to the lower ng/L range (Tamagawa et al., 2006). A small fraction (estimated at less than 10%) of estrogens is removed through the standard WWTP process, the majority being released in the environment either dissolved in the effluent or adsorbed to the sludge, which will result in soil and groundwater contamination when the sludge is used as fertilizer. However, there are huge discrepancies from one study to another concerning the removal rates of EDCs, probably due to different treatment processes among WWTP (Auriol et al., 2006).

Bioremediation, a process using exogenous microorganisms to remove EDCs from contaminated environments, is often difficult to control. Bioremediation carries with it many inherent difficulties, such as maintaining the microbial population necessary to degrade a certain compound, and the necessary growth conditions for the required microbe. Some microbes may require specific conditions difficult to optimize in the field. Also, the nutrients necessary to these microorganisms increase the Chemical Oxygen Demand of the WWTP (Tanaka et al., 2000). These factors can limit the overall success of bioremediation.

There also exist several physical and chemical treatment methods for the removal of EDCs from contaminated environment, all somewhat unsatisfactory. Desorption under vacuum is a physical method used for remediation of the environment polluted by toxic chemicals but is limited to volatile compounds. Nanofiltration, adsorption on hydrophobic membranes or powdered activated carbon, coagulants such as aluminium and ferric salts can also be used to remove trace contaminants. Chlorination is not a method of choice since the resulting chlorinated products have carcinogenicity and/or mutagenicity. Ozonization is effective in oxidizing the EDCs but, since it is non selective, the generated radical HO. is inefficiently consumed by other harmless compounds, increasing the consumption of ozone; moreover, the products generated by ozonization of EDCs are unknown (Tanaka et al., 2000, Auriol et al., 2006).

In contrast, bioprocesses involving enzymes as catalysts have several advantages over conventional biological, chemical and physical treatment processes. They are cost-competitive technological options with attractive characteristics such as low energy requirements, easy process control and vast operability over a wide range of environmental conditions as enzymes are able to act in a wide range of pHs, temperatures and ionic strengths and remain active even if these conditions quickly change. Enzymatic processes also have high specificity towards pollutants, high reaction rates, and contribute to the reduction in sludge volume.

Bioprocesses offer other advantages over microbial processes due to their specificity. On one hand, bioprocesses have more predictability, as an enzyme will always generate the same product from the same substrate. This substrate and product specificity of enzymes also enables enzymatic decontamination to be run together with other processes such as microbial ones. Product specificity ensures that no toxic co-products are generated in suboptimal conditions (Cabana et al., 2007, Ikehata et al., 2004).

On the other hand, enzymes with a high specificity will only degrade a specific target compound even if more easily degradable compounds are present, while microbes will often first metabolize those compounds that are easiest for them to degrade before degrading more resilient compounds. Thus bioprocesses are more effective than microbial processes in decontamination project when the compound of interest is recalcitrant.

When several contaminants are present, enzymes with less specificity can be used to degrade concomitantly all the contaminants; it is also possible to use a mix of different enzymes to degrade various compounds simultaneously. Finally, because most enzymes have high affinity for their substrates, they are effective even at very low concentration of their substrates; this characteristic is particularly well suited for bioremediation of EDCs.

Bioprocesses are also more durable and versatile, especially when the enzymes are immobilized. Advantageously, in addition to improving the enzymes' stability thus increasing their resistance to harsh environments, immobilization facilitates their recovery and reuse, thus lowering operational costs (Lloret et al., 2012b,c). Bioprocesses can easily be adapted to several types of treatment processes such as membrane bioreactors with the enzyme being immobilized on the membrane (Mita et al., 2010, Lloret et al., 2012a), packed or fluidized bed reactors with the enzyme being immobilized on a resin.

Finally, there is a very pragmatic benefit of using enzymatic treatment as enzymes are biodegradable. Thus, the enzymes that are not recovered will simply degrade in the environment after they are no longer needed. And, unlike other remediation methods, there is no buildup of biomass or added chemicals that must be removed afterwards (Ruggaber et al. 2006).

1-Introduction

1.2 Objective

Protéus has built a collection of more than 350 industrial enzymes for white biotechnology applications. Fuelled by the high public and political concern about EDCs in the environment and their impact on human health, we wanted to screen our collection for efficient EDC-degrading enzymes.

We targeted three families of EDCs: estrogens (E1, E2, E3, EE2, ßE2) since they are the most potent EDCs, BPA because it can be present at high concentrations in surface and marine water (up to 21mg/L) and phthalates since they are produced in extremely large volumes (400 to 500 thousand tons per annum in Europe) (Harris et al., 1997).

Estrogens and BPA are known to be degraded by laccases (phenol oxidases), yielding products with largely reduced endocrine disrupting potency (Suzuki et al., 2003). Structures of these EDCs are shown in Figure 1, exhibiting phenol groups (estrogens and BPA) that are targets for laccases. In some cases, laccase efficiency and target spectrum can be improved by using so-called mediators, which are phenolic compounds (Lloret et al., 2010).

Phtalates are targets for esterases and lipases, via their ester groups (see Figure 1) (Mita et al., 2010, Niazi et al., 2001).

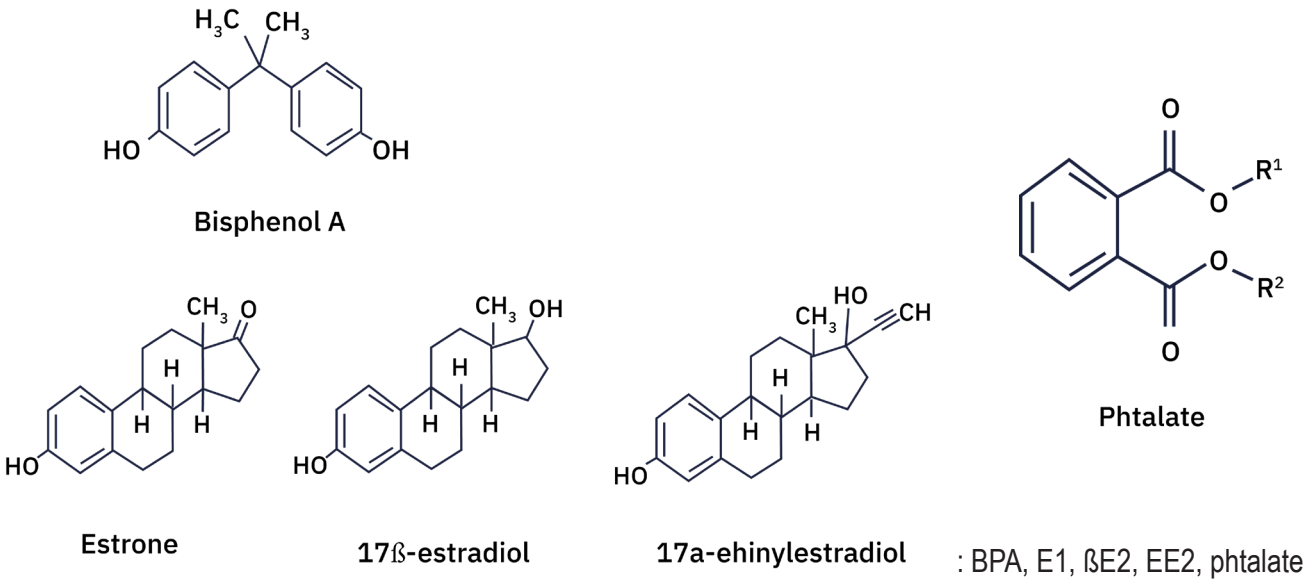

Figure 1

2-Material and methods

2.1 Chemicals

All chemical compounds are from Sigma-Aldrich.

2.2 Enzymes

The following enzymes from Proteus collection were tested:

- 10 laccases (E542, E556, E557, E561, E3215, E3610, E3661, E3919, E3910, E3918)
- 3 enzymatic cocktails (CEP4, CEP10, CEP11)
- 64 esterases and lipases

2.3 Endocrine disrupting activity assay

The endocrine disrupting (ED) activity assay LYES was performed according to Tsutsumi et al. (2001). The modified Saccharomyces cerevisiae strain was kindly provided by Dr Choi.

Due to the lower ED activity of BPA compared to estrogens, the contact time between modified Saccharomyces cerevisiae strain and BPA has been extended to one night, compared to 4 hours for estrogens.

2.4 Enzymatic degradation assays

The concentrations of EDCs in the assays have been chosen so as to have a similar ED activity whatever the compound. As a consequence, E1, E2 and EE2 concentrations are 400ng/mL, E3 40µg/mL, BPA 0.75mg/mL and phthalates concentrations are 0.1mM for BBP and 2mM for DIBP. Assays have been performed at pH 4 and 7.

The modified Saccharomyces cerevisiae strain has been cultivated over night at 28°C.

EDCs have been incubated at 30°C with various enzyme and mediator concentrations as described below in microtiter plate in a final volume of 200µL. After different treatment times, 2.5 or 10µL reaction samples have been used for ED activity assay.

3-Results and discussion

3.1 Endocrine disrupting activity of targeted compounds

The LYES assay was performed on serial dilutions of each of the targeted compounds. The detection threshold and linear range obtained are shown in Table 1.

COMPOUND	DETECTION THRESHOLD	LINEAR RANGE
E1	6 ng/mL	6-200 ng/mL
E2	6 ng/mL	6-200 ng/mL
EE2	6 ng/mL	6-200 ng/mL
E3	1.25 µg/mL	1.25-40 µg/mL
BPA	10 µg/mL (*)	10-600 µg/mL (*)
BBP	0.6 µg/mL	4-40 µg/mL
BPA	14 µg/mL	560 µg/mL

Table 1. Detection threshold and linear range of endocrine disrupting activity measurement with the LYES assay

(*) due to low activity of BPA, the activity assay conditions have had to be modified

3.2 Estrogenic compound degradation

E1, E2 and EE2 (400ng/mL) and E3 (40µg/mL) were incubated at pH4, 28°C for 24h each with 5 different laccases and 3 different enzymatic cocktails alternately. The LYES assay has then been performed. The % residual endocrine disrupting activity has then been calculated by comparison with a negative control (no enzyme). See Figure 2.

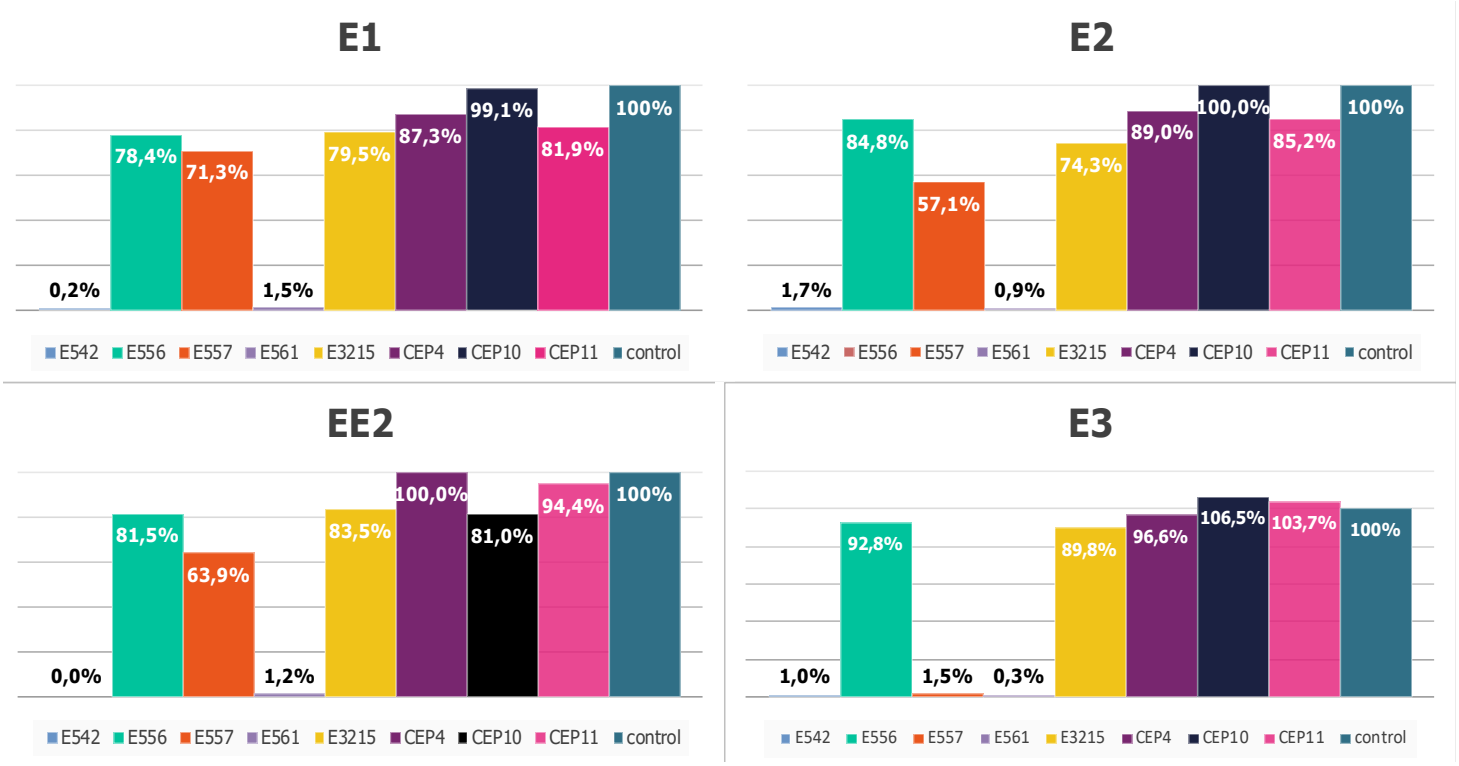

Figure 2. Enzyme screening on estrogenic compounds at pH 4.

Complete abatement of ED activity is obtained for all four compounds by 2 of the laccases: E542 and E561. Additionally, E3 is also inactivated by E557. The other laccases and the enzymatic cocktails have no significant activity on these compounds.

These results show the ability of laccases to abolish endocrine disrupting activity of estrogenic compounds. However, all the enzymes evaluated in the screening have an acidic optimum pH which is not well suited for water treatment. Even if the optimum pH of these enzymes is between 3 and 5, checking their activity at the non-optimal pH 7 was thus necessary. Moreover, the oxidizing activity towards substrates that are non-natural to laccases is known to be improved by so-called mediators. Common mediators are phenolic compounds, which are substrates of laccases such as ABTS or syringaldazine. However, for the targeted application (water treatment), they cannot be used because of their toxicity and high cost. We thus looked for non-conventional biosourced, low-cost and non-toxic mediators, and selected two: Mediators 1 and 2. The enzymes that were initially screened at pH 4 were thus retested at pH 7 without or with several concentrations of one of those mediators (mediator 1) (150, 300, 450 or 600 mg/L). The ED activity was measured at different time points during incubation. The only enzyme showing a significant effect on the four estrogenic compounds at pH 7 is E0557. See Figure 3. The results show a strong improvement with the use of the mediator, even at the lowest concentration of 150mg/L. The ED activity of E1 and E3 is fully removed in 7h, wherever 90% of E2 and 85% of EE2 activities are removed in 24h and 150mg/L of mediator 1.

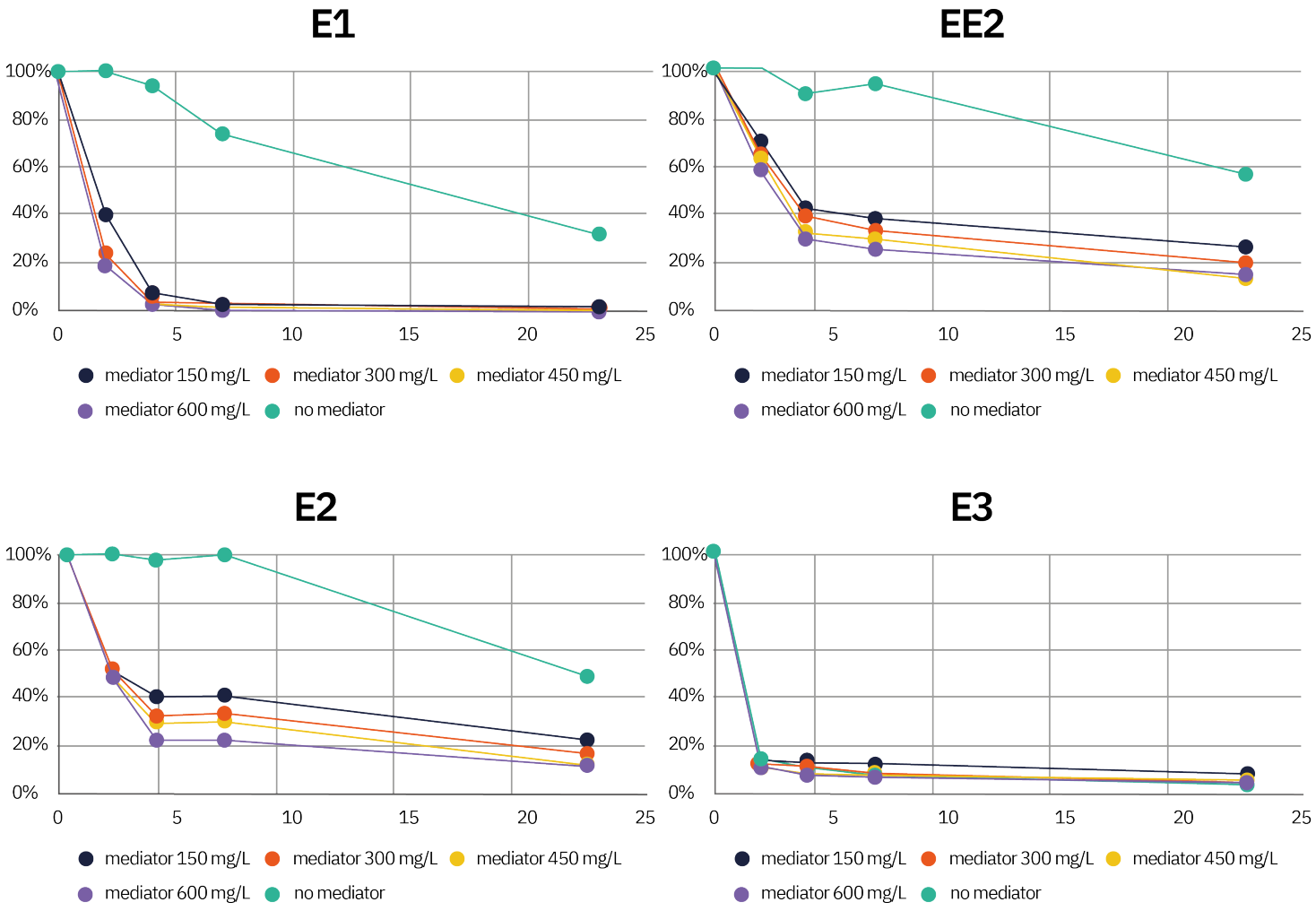

Figure 3. Effect of E0557 on estrogenic compounds at pH7 with or without mediator 1

Mediators 1 and 2 were also tested in parallel at concentrations from 1 to 8 g/L. Laccase E0557 in combination with 1g/L of mediator 2 allowed an abatement of more than 98% of the ED activity of all 4 estrogenic compounds in less than 1 hour (data not shown).

Complementary to the previous approach that consisted of making laccases work at a non-optimal pH, we also looked for laccases having a good activity at neutral pH. Three laccases (E3910, E3918 and E3919) have been produced and tested as previously on estrogenic compounds but at pH 7 instead of 4. E3918 is the only one diminishing significantly the ED activity of all four compounds. However, this abatement is not total contrary to those obtained with acidophilic enzymes.

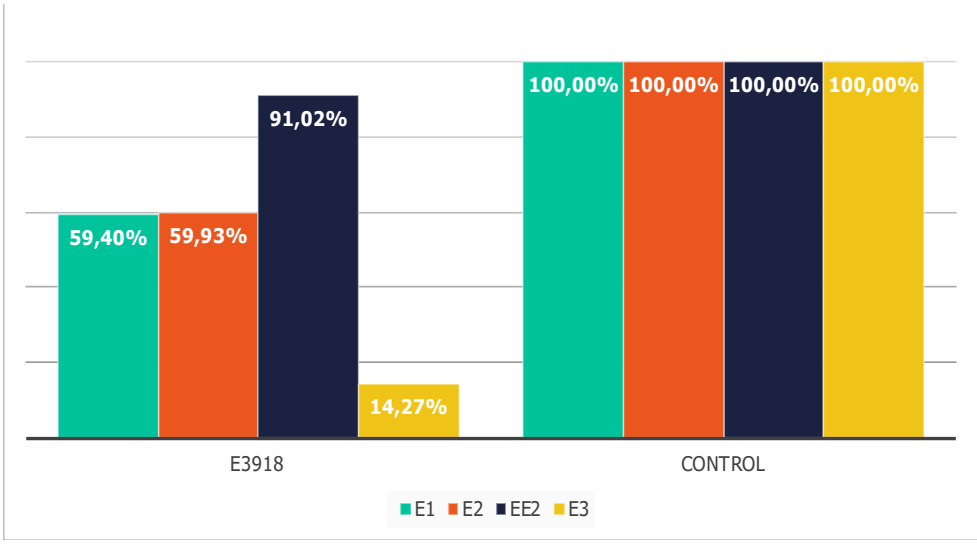

Figure 4. Effect of E3918 on estrogenic compounds at pH 7.

Since E3918 has a significant effect on the four EDC, we tested the effect of two different natural mediators (mediator 1 and mediator 2) at 1g/L final concentration. The ED activity was measured at different time points during incubation. The results show a strong improvement with the use of the mediators and particularly mediator 1. The ED activity of E1 is fully removed in 4h, wherever 70 to 80% of the other 3 compounds are removed in 24h. See Figure 5.

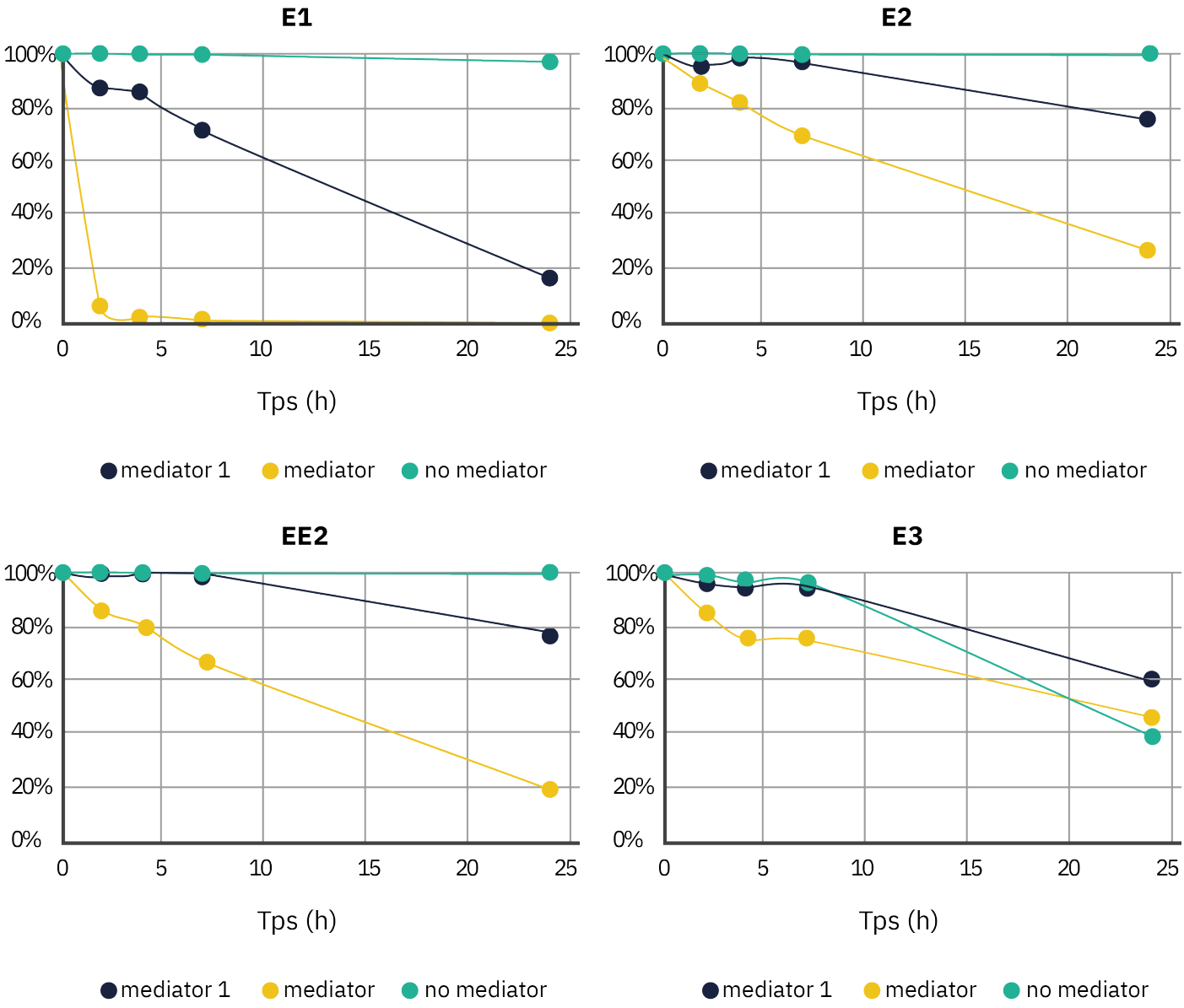

Figure 5. Effect of E3918 on estrogenic compounds at pH7 with or without mediator

From the above results, we can see that the E0557 laccase with mediator 1 and the E3918 laccase with mediator 2 have promising activity on the four tested estrogenic compounds. Moreover, it is worth noting that we used high concentrations of EDCs (400ng/L for E1, E2 and EE2, 40µg/L of E3) to be in the dynamic range of the LYES assay. These concentrations are much higher than the concentrations generally measured in WWTP effluents: up to 76, 48, 7.5 and 21 for E1, E2, EE2 and E3, respectively (Auriol et al., 2006).

Bisphenol A is also known to be a target for laccases. Since it would be interesting to obtain a wide spectrum decontaminating solution against EDC, we tested both these enzymes on Bisphenol A.

3.3 Bisphenol A degradation

Laccases E0557 and E3918 have been evaluated to treat Bisphenol A at a concentration of 0.75mg/mL. The ED activity was measured at different time points during incubation at pH7 without mediator. The results show more than 99% abatement of ED activity in 7h with E0557 and in 24h with E3918. See Figure 6.

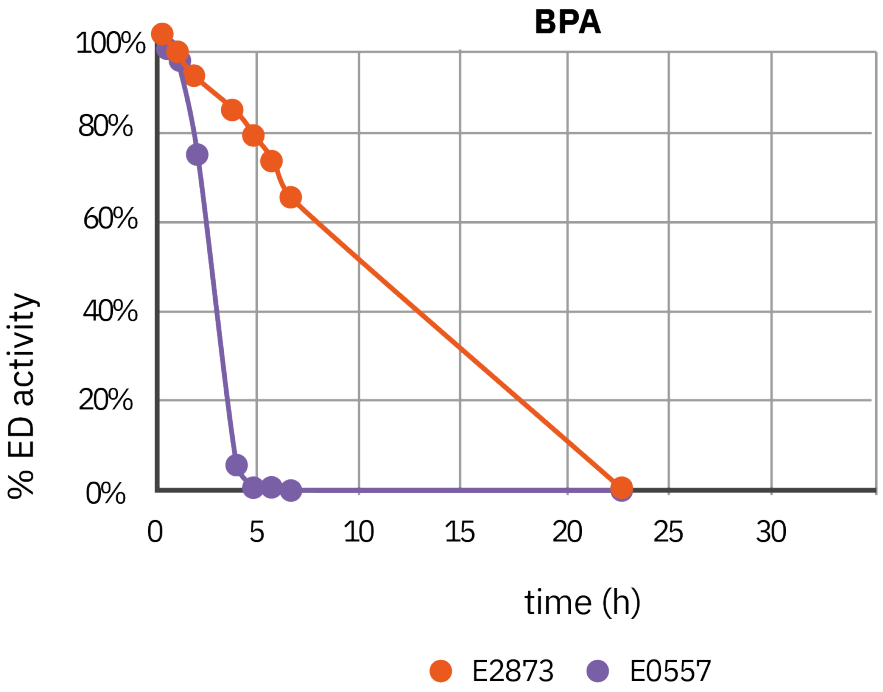

Figure 6. Effect of E0557 and E3918 on BPA at pH7 without mediator

3.4 Phtalate esters degradation

A first screening of esterases and lipases from Proteus collection (64 enzymes) was performed on two different phthalates (BBP and DIBP) at 0.1mM final concentration (approximately 30µg/mL) at pH7. Degradation of these compounds was evaluated using the LYES assay at t0 and t24h and compared with negative controls. Four enzymes among the 64 gave significant abatement of ED activity of both substrates: E3736, E3749, E3766 and E3957. The ED activity of these enzymes were further assayed at different time points during incubation. The results are shown in Figure 7. Three enzymes (E3736, E3766 and E3957) gave more than 95% reduction of ED activity in 1.5h on BBP. These three enzymes were then evaluated on DIBP and gave more than 80% reduction of ED activity in 1.5h (see Figure 7).

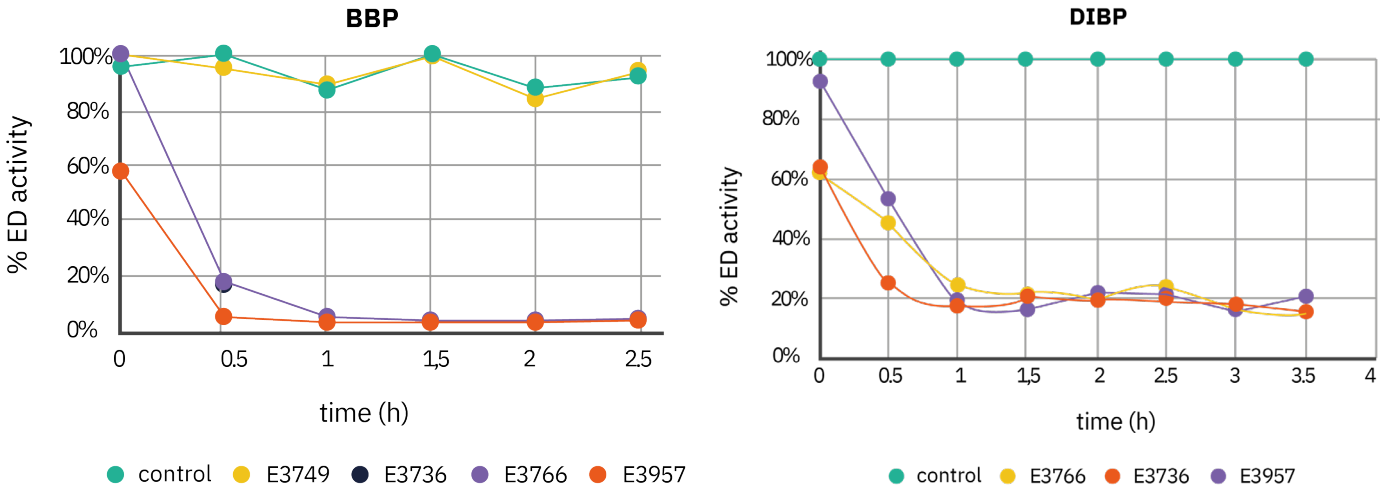

Figure 7. Effect of esterases on phthalate esters

4-Conclusions and perspectives

We have identified a laccase (E0557) that has a very efficient removal of ED activity of bisphenol A and, in combination with mediator 1, of all four estrogenic compounds tested at pH 7 and 28°C. Moreover, we also have identified three esterases that strongly decrease ED activity of phthalate esters in the same conditions. These conditions are environmentally relevant for water treatment operations.

These enzymes are available at Protéus for testing. We can also offer our expertise for any relevant problem solving you may require for implementing innovative and efficient process solutions.

Because enzymes do not interact with each other we could also imagine mixing the identified laccase and esterase with other enzymes to widen the range of pollutants to be decontaminated such as dehalogenases targeting organochlorinated compounds.

Proteus has developed proprietary enzyme improvement technologies named Evosight™ and L-shuffling™ (Fourage et al., 2010) that can be used, for example, to increase the activity of an enzyme, thus diminishing the amount of enzyme necessary to obtain a given decontamination rate and thus again diminishing the treatment cost.

Finally, Proteus has a strong expertise in enzyme immobilization and is in a position to develop an immobilization support suitable for water treatment, with the suitable enzymes either individually or mixed.

Consequently, our results lay the foundations for the development of adapted industrial processes for the degradation of endocrine disrupting compounds.

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