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Inorganic oxide systems as platforms for synergistic adsorption and enzymatic conversion of estrogens from aqueous solutions: Mechanism, stability and toxicity studies

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ABSTRACT

Synthetic hormones constitute a dangerous class of pollutants as they impose risks on the reproductive health of ecosystem life and humans via the water, including drinking water. Traditional methods of wastewater treatment seem to be inefficient. In this work, design and fabrication of a new biosystem made of CaSiO$_3$ and laccase is reported and its application for removal of 17β-ethynylestradiol (EE2) from aqueous systems is demonstrated. EE2 is a synthetic estrogen, which is known to resist degradation. The effect of treatment time, pH, temperature, estrogen concentration and mass of the biocatalytic system was investigated. Treatment of an EE2 solution (0.1 mg/L) with 100 mg of the biosystem for 12 h at pH 5, 25°C resulted in 100% removal efficiency. The data confirmed that the EE2 was degraded synergistically by simultaneous adsorption and biocatalytic conversion, with the enzymatic conversion being dominating. The efficiency of estrogen removal by the biosystem varied depending on type of cations and anions present in the solution. After 10 cycles of repeated use, and 20 days of storage, the CaSiO$_3$-laccase biosystem retained ~40% of its initial activity. Application of the CaSiO$_3$-laccase biosystem significantly reduced toxicity and estrogenic activity of the solution. Finally, it was also possible to remove more than 40% of EE2 from samples of real wastewater using the CaSiO$_3$-laccase biosystem. The method may pave the way for new efficient approaches for removal of pharmaceuticals and hormones from wastewater.

1. Introduction

The presence of pharmaceuticals, hormones, and pesticides in the environment pose a threat to organisms due to their toxic effects on living cells. In aquatic wildlife, increasing concentrations of both, natural and synthetic hormones cause feminization of male fish, mainly by reducing the size of the testes [4,66,74], and such hormones may change the reproductive performance [53] and negatively affect other reproductive characteristics, as well as the health state of the aquatic ecosystem life in general [20,56,61,68].

While estrogens are necessary for the proper functioning of the human body, their accumulation and consumption above a safe limit can cause negative health effects. These effects range from inducing premature menopause, interfering with the development of the reproductive system, causing virilization in young women, to increasing breast cancer risk in women [41], prostate cancer in men [42], and elevating cardiovascular disease risk [73].

Due to the fact that estrogens are quite resistant to classical methods of remediation [37], new water remediation processes are needed for effective removal of these compounds from wastewater [51,52]. One of the promising methods of estrogens removal from aqueous solutions seems to be enzymatic, oxidative conversion supported by adsorption [80]. Immobilized oxidoreductases are more stable than the free, soluble enzymes over a wide range of various process conditions and reusability of the catalyst is moreover improved. Products of enzymatic conversion are less toxic than their substrates, but should preferably be removed [27]. Laccase immobilized on beads and used in a fluidized bed system, has been shown to efficiently remove alkylphenolic endocrine disruptors.

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in model wastewater [13], and laccase immobilized in membranes [80] or on electrospun materials [18] has proven efficient for removal of significant amounts of tetracycline (>80% at test levels of 1 mg/L) from model waste water samples. Adsorption is less selective than enzymatic conversion, but it may increase the efficiency of removal. Shao et al., [57] studied synergistic removal of antibiotics using laccase immobilized onto hollow mesoporous carbon spheres. The adsorption efficiency equaled 55% and 70% for tetracycline and ciprofloxacin, respectively, but additional laccase action made it possible to remove 99% and 97% of these antibiotics. Likewise, Xu et al., [76] used horseradish peroxidase immobilized onto nanofibrous membranes to catalyse removal of paraacetamol, and while removal via adsorption on the membranes was ~15%, the enzymatic conversion equaled 83%, resulting in 98% total removal efficiency. However, despite these studies, synergistic removal of endocrine disrupting chemicals by simultaneous biocatalytic conversion and adsorption is neglected.

The present work introduces a new concept for removal of hormones from wastewater, namely a novel biosystem comprising CaSiO$_3$ combined with laccase. CaSiO$_3$ is suitable for enzyme immobilization mainly due to its high thermal and chemical stability, porosity, well-developed surface area as well as the presence of numerous hydroxyl groups that facilitate simple enzyme binding. CaSiO$_3$ is moreover commercially available and relatively inexpensive making it ideal for large-scale application. In addition, due to its sorption ability and porous structure, the CaSiO$_3$ material enables both immobilization of the enzyme and simultaneous/additional adsorption of pollutants and their conversion products. The use of the CaSiO$_3$-laccase biosystem made it possible to compare the efficiency of removal of 17α-ethinylestradiol (EE2) by simultaneous adsorption and biocatalytic conversion, and the effect of various process conditions, including time, pH, temperature, estrogen concentration, mass of the biosystem and presence of cations and anions in the initial EE2 solution, was investigated. The stability of the biosystem was studied in terms of its reusability and storage stability, and toxicity and estrogenic activity studies were conducted to compare the nature of solutions before and after the biocatalytic conversion. The experimental work moreover included validation of the workability of the CaSiO$_3$-laccase biosystem in two types of genuine wastewater samples. The study provides a proof-of-concept for production of an inexpensive, efficient biocatalytic system for removal of hormone-like micropollutants from water and demonstrates elimination of toxicity and estrogenic activity.

2. Materials and methods

2.1. Chemicals and materials

Calcium silicate (CaSiO$_3$), laccase from Trametes versicolor (EC 1.10.3.2; $>0.5$ U/mg), Bradford reagent, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diaminonium salt (ABTS), sodium acetate, 50 mM phosphate buffer and 50 mM acetate buffer solutions, 17α-ethinylestradiol (EE2), N,O-bis(trimethylsilyl)trifluoroacetamide + 1% trimethylsilyl chloride (BSTFA+1% TMCS), dimethylformamide (DMF), lead(II) nitrate, zinc chloride, iron(II) chloride, nickel(II) chloride, calcium chloride, magnesium chloride, sodium chloride, potassium chloride, ammonium chloride, silver chloride, sodium sulfate, sodium bromide and sodium cyanide solutions at concentrations of 50 mg/L [21,46,50,10]. Mixtures were placed in an IKA KS 4000i control incubator (IKA Werke GmbH, Germany) and were sampled every specified period of time. The solution samples after reactions were subjected to GC-MS analysis.

In the study on EE2 removal from real wastewater, 10 mL of two various solutions were used: (i) entering the wastewater treatment plant (before treatment) and (ii) leaving the treatment plant (after treatment). To such a solution 12 mg of free laccase and corresponding amount of active and inactive laccase immobilized onto CaSiO$_3$ material, was added. The systems were covered and placed in an IKA KS 4000i control incubator (IKA Werke GmbH, Germany) for 12 h and were sampled every specified period of time. The solution samples after reactions were subjected to GC-MS analysis.

The concentration of the EE2 in the solutions after removal process was determined based on gas chromatography results, using standard calibration curve for the solutions at known concentration. The removal efficiency ($R_e$) of EE2 was calculated based on Eq. 1:

$$R_e = \frac{C_i - C_f}{C_i} \times 100\%$$

where $C_i$ and $C_f$ denote the initial and final concentrations of EE2, respectively.

2.2. Preparation of CaSiO$_3$-laccase systems comprising active and inactive biomolecules

In order to immobilize laccase onto CaSiO$_3$, 100 mg of calcium silicate was placed into vials and mixed with 2 mL of laccase solution (10 mg/mL in acetate buffer at pH 5) at 25 °C for 24 h using an IKA KS 4000i control incubator (IKA Werke GmbH, Germany). After immobilization process, the support was centrifuged using LLG unIFUGE 5 (LLG Labware, Ireland) at 4000 rpm and washed 3 times with acetate buffer (pH 5). For preparation of CaSiO$_3$-inactivated laccase biosystem, 100 mg of CaSiO$_3$ with immobilized laccase was placed in an IKA KS 4000i control incubator (IKA Werke GmbH, Germany) at 80 °C for 5 h to thermally inactivate the enzyme.

2.3. Removal of estrogen (EE2)

12 mg of free laccase, which corresponds to the amount of the biomolecule adsorbed onto CaSiO$_3$, pure CaSiO$_3$ and CaSiO$_3$ with active and inactivated laccase, were used for EE2 removal at various process conditions. The effect of various reaction time was studied within time range 1–12 h at pH 5, 25 °C, EE2 concentration of 1 mg/L, using 100 mg of biosystems. The effect of pH was determined over pH ranging from 3 to 8, at 25 °C, EE2 concentration of 1 mg/L, using 100 mg of biosystems and for 12 h. Effect of estrogen concentration on its removal rate was determined within 12 h at pH 5, 25 °C, using 100 mg of biosystems and tested EE2 solutions at concentrations from 0.1 to 5.0 mg/L. Finally, the effect of biosystems dose (50–500 mg) was examined at pH 5, 25 °C, EE2 concentration of 1 mg/L and for 12 h. The removal efficiency of EE2 in the presence of competitive cations and anions was determined by incubating 12 mg of free laccase and 100 mg of CaSiO$_3$-laccase biosystem or CaSiO$_3$-inactivated laccase, containing 12 mg of the enzyme, separately, in 1 mg/L solution of estrogen at pH 5, 25 °C and for 12 h. The tests were performed in the presence of lead nitrate, zinc chloride, iron(II) chloride, nickel(II) chloride, calcium chloride, magnesium chloride, sodium chloride, potassium chloride, ammonium chloride, silver chloride, sodium sulfate, sodium fluoride, sodium bromide and sodium cyanide solutions at concentrations of 50 mg/L [21,46,50,10]. Mixtures were placed in an IKA KS 4000i control incubator (IKA Werke GmbH, Germany) and were sampled every specified period of time. The solution samples after reactions were subjected to GC-MS analysis.

The reusability of CaSiO$_3$-laccase biosystem and CaSiO$_3$-inactivated laccase was studied over 10 consecutive catalytic cycles of EE2 removal. The removal efficiency was calculated from the final concentration of EE2 after processing at pH 5, 25 °C, at estrogen concentration of 1 mg/L and for 12 h. After each cycle, the biosystem was separated from the reaction mixture by centrifugation at 4000 rpm, washed by acetate buffer at pH 5 and placed in the estrogen solution (initial concentration 1 mg/L). The solution samples after reactions were analyzed by GC-MS. Amount of the enzyme eluted from the CaSiO$_3$-laccase biosystem after...
each repeated catalytic cycle was assessed by the Bradford protein analysis using bovine serum albumin as a standard [11].

In order to investigate the storage stability of the free enzyme and biosystem made of CaSiO\textsubscript{3} and active laccase, 12 mg of free oxidoreductase and 100 mg of CaSiO\textsubscript{3}-laccase biosystem, were stored at 4 °C for 20 days. To measure relative activity of enzymes, the model reaction with 0.05 mM of ABTS (λ = 420 nm) was carried out for 10 min, at pH 5 and 25 °C. The initial activity of the free laccase and tested biosystem was defined as 100% activity.

2.5. Amount of immobilized enzyme

Amount of laccase immobilized onto CaSiO\textsubscript{3} and immobilization yield was determined using the Bradford method [11]. 0.5 mL of solution after immobilization or 0.5 mL of solution after each biocatalytic cycle were mixed with 0.5 mL of Bradford reagent. After 5 min, the absorbance at 595 nm was measured. The laccase concentration was determined using bovine serum albumin as a standard. The relative amount of the supported enzyme was calculated by considering initial laccase concentration, laccase concentration in the solution after immobilization and laccase concentration in the solution after each of the consecutive catalytic cycle.

2.6. Toxicity and estrogenic activity analysis

Toxicity of EE2 solutions, before and after removal process, was estimated based on the investigation of the mortality of Artemia salina [36]. Briefly, 500 mg of Artemia salina eggs were placed in 500 mL of NaCl solution at concentration of 25 g/L and incubated at 25 °C for 24 h, with exposure to permanent lighting. Next, the hatched larvae were placed in the EE2 solutions, before and after removal process, and left for 24 h at 25 °C. The mortality of Artemia salina was calculated based on Eq. 2:

$$MO(\%) = \frac{A_d}{A_i} \times 100\%$$ (2)

where MO denotes the mortality of Artemia salina (%), and \(A_d\) and \(A_i\) represent number of dead larvae and initial number of larvae, respectively.

To study the estrogenic activity of EE2 solutions before and after the removal process, S-YES\textsuperscript{MD} biological measurement system, based on genetically modified yeast Saccharomyces cerevisiae, was used. The measurement of estrogenic activity relies on the dependence of the amount of the reporter enzyme β-galactosidase on the concentration of estrogen. The reporter enzyme activity (EA) was measured spectrophotometrically (Multiskan SkyHigh microplate spectrophotometer, Thermo Fisher Scientific, USA) and calculated based on Eq. 3 [19]:

$$EA = \frac{A_{580nm(sample)}}{A_{580nm(blank)}} \times \frac{A_{620nm(sample)}}{A_{620nm(blank)}}$$ (3)

where A means absorbance of samples or blank measured at specific wavelength.

Based on EA calculations, it was also possible to estimate the relative estrogenic activity (REA), based on Eq. 4 [8]:

$$REA = \frac{EA_{before} - EA_{after}}{EA_{before}}$$ (4)

Fig. 1. CLSM photographs of: (a) CaSiO\textsubscript{3} and (b) CaSiO\textsubscript{3}-laccase in reflection mode (left) and fluorescence mode (right).
where \( \text{REA} \) denotes relative enzyme activity, \( \text{EA} \) denotes enzyme activity and \( \text{IE} \) means the interval of estrogenicity between the highest reporter enzyme activity for 17\( \beta \)-estradiol (E2) as a reference standard and the reporter enzyme activity for blank sample. Calculations of estrogenic activity were made using BioVAL® software supplied by new_, diagnostics GmbH (Germany).

### 2.7. Analytical procedures

A confocal laser scanning microscope LSM710 (Zeiss, Germany) with an argon laser (488 nm) was used to determine morphology of CaSiO\(_3\) material before and after laccase immobilization. The photographs were made in material and fluorescence modes using the laser operated at 458 and 488 nm, respectively. ASAP 2020 physisorption analyzer (Micromeritics Instrument Co., USA) was used to determine the parameters of the porous structure (Brunauer–Emmett–Teller (BET) surface area, average pore diameter and total pore volume, calculated based on the Barrett–Joyner–Halenda (BHU) algorithm) of pure CaSiO\(_3\) and biosystem with immobilized laccase. Prior to the measurement, samples were degassed at 120 °C. Furthermore, they were analyzed using low-temperature sorption of \( \text{N}_2 \) (−196 °C). X-ray microanalysis (EDS) was carried out using the EDS analyzer contained in the Tescan (Czech Republic) scanning electron microscope with Gamma-Tec tooling from Princeton Inc. Fourier transform infrared spectra (FTIR) of CaSiO\(_3\), before and after laccase immobilization, were obtained using a Bruker Vertex 70 spectrometer (Bruker, Germany) in the attenuated total reflectance (ATR) mode. The spectroscopic measurements performed in the study were carried out using a UV-1280 spectrophotometer (Shimadzu Corp., Japan) and Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific, USA).

To measure the degradation efficiency of EE2, gas chromatography analysis was used. The samples were lyophilized (Alpha 1.4 LD plus, Christ, Germany) for 96 h at 0.36 mbar and at −30 °C. The residue was then resuspended in 100 µL of DMF and transferred to chromatographic vials. The samples were derivatized by adding 100 µL of BSTFA+ 1% TMCS, and then heated at 65 °C for 2 h. Furthermore, they were subjected to chromatographic analysis. Quantitative analysis of the tested compounds was carried out on a Pegasus 4D gas chromatograph (Leco, USA) with a BPX-5 column (28 m × 250 µm × 0.25 µm) and helium as the carrier gas. The sample (1 µL) was dispensed on the inlet at a temperature of 250 °C. The chromatograph was operated in the programmed temperature increase mode: 80 °C for 1 min, increasing by 20 °C/min to 200 °C, then increasing by 8 °C/min to 280 °C and maintaining the final temperature for 3 min. The chromatograph was coupled to a mass spectrometer (TOF-MS), which conducted analysis of the eluent from the column using an ion source operating in a positive ion mode. The temperature of the ion source was 250 °C and the energy was 70 eV. Quantitative analyses were conducted for ions 73, 196, 232, 285 and 425 in masses, based on standard solutions. Chroma TOF-GC v4.51.6.0 software was used for the data analysis. All experiments were carried out in triplicate and the results are shown as a mean value.

### 3. Results and discussion

#### 3.1. Characterization of CaSiO\(_3\)-laccase biosystem

The morphology and structure of the CaSiO\(_3\) oxide material, before and after immobilization, are presented in the confocal laser scanning images (CLSM) images (Fig. 1). It could be seen that support material before laccase immobilization is characterized by densely packed, spherical particles, which form aggregates less than 1 µm in size (Fig. 1a). On the other hand, after immobilization process it exhibits significant changes in the structure and morphology. Aggregates with sizes larger than in the case of pure CaSiO\(_3\) material (at around 1.5 µm) are clearly seen (Fig. 1b). Moreover, the bright points visible in the CLSM image of CaSiO\(_3\)-laccase system in fluorescence mode, characteristic for proteins, confirm that the immobilization was efficient [23]. Similar observations were made by Anwar et al. [3], who immobilized lipase onto SnO\(_2\) hollow nanotubes. They observed bright points on a CLSM photo of a sample after enzyme immobilization, and explained that they raise from lipase presence on the SnO\(_2\) surface.

Moreover, an energy dispersive spectroscopy (EDS) elemental analysis was made to confirm the laccase immobilization onto the CaSiO\(_3\) surface (Fig. 1). Due to the fact that laccase is a multicopper oxidase, an increase in the mass contribution of nitrogen and copper in the CaSiO\(_3\) sample after laccase immobilization was noted as compared to the pure material, corroborating the attachment of the enzyme to the support. Further, the value of the surface area, pore volume and average pore size of the tested material, before and after laccase immobilization, changed significantly. For calcium silicate, the surface area, pore volume and average pore diameter were initially 33 m\(^2\)/g, 0.14 cm\(^3\)/g, and 27.3 nm, respectively, whereas after laccase immobilization process it decreased to 28 m\(^2\)/g, 0.13 cm\(^3\)/g, and 23.5 nm, respectively. These changes are caused by adsorption of the enzymes onto the surface and into the CaSiO\(_3\) pores as a result of the immobilization process and therefore prove the presence of the enzyme in the fabricated biocatalyst structure [22,23,34].

To verify the effective laccase immobilization and to characterize specific functional groups of the obtained biosystems, FTIR analysis was also made (Fig. 2). It can be seen that the CaSiO\(_3\) and CaSiO\(_3\)-laccase spectra possess signals in the ranges of 1100–860 cm\(^{-1}\) and 600–550 cm\(^{-1}\), which are characteristic for symmetric stretching vibration of the O–Si–O, symmetric stretching vibration of the Si–O–Si bridges and stretching vibration of Si–O bonds [6,9]. The signal around 1450 cm\(^{-1}\) is characteristic for the stretching vibration of bonds including Ca\(^{2+}\) ions. In the FTIR spectrum of the sample upon immobilization, additional new signals were noted.

Apart from signals characteristic for CaSiO\(_3\) support, wide band in the range of 3600–3100 cm\(^{-1}\), related to the stretching vibration of the –OH and –NH group, as well as signal at 2900 cm\(^{-1}\) ascribed to stretching vibration of the C–H bond, specific to enzyme structure, can be seen. The presence of signals at 1650 and 1540 cm\(^{-1}\) ultimately confirms the effectiveness of the laccase immobilization onto CaSiO\(_3\) material [12,81].

![FTIR spectra of CaSiO\(_3\) and CaSiO\(_3\)-laccase system.](image-url)
3.2. Removal of 17α-ethinylestradiol

3.2.1. Reusability and storage stability of produced biosystems

The reusability study was carried out to investigate the possible reuse of the CaSiO$_3$-laccase biosystem for removal of EE2, and hence to test possible applicability of the proposed biocatalyst (Fig. 3a). It was observed that increasing number of catalytic cycles decreases the removal efficiency. After 5th cycle, the removal efficiency reached around 75% and after 10th cycle it equaled to 32%. This fact may be related to the partial elution of the enzyme from the support or laccase inhibition by conversion products [17]. In case of amount of immobilized enzyme, it was noted that the initial amount of laccase adsorbed onto CaSiO$_3$ equaled 12 mg, and the relative amount of biomolecule onto the support dropped with increased number of repeated uses. However, it should be emphasized that after 5 removal cycles, both, removal efficiency of estrogen and the relative amount of immobilized laccase were around 80%, which shows possibility of efficient reuse of the proposed biosystem. The obtained results confirmed the efficient laccase immobilization and enzyme attachment to the surface of the support mainly by physical interactions such as van der Waals forces, ionic interactions and hydrogen bonding due to simple suspension of the support in enzyme solution and lack of support modification [24]. On the one hand, this type of interactions reduces the possibility of enzyme structure disruption upon immobilization and facilitates high activity recovery. On the other hand, weak enzyme-support interactions lead to relatively rapid leaching of the enzyme from the support (over 50% of the enzyme was eluted after 8 repeated cycles) that resulted in decreasing removal efficiency over repeated use (Fig. 3a). According to Zofair et al. [84], the decrease of enzyme activity during the reusability tests is also related to the partial leaching of the enzyme from the support as well as slight enzyme inactivation over repeated use, which stays in agreement with presented data. Nevertheless, retention of over 80% of removal efficiency after 5 repeated cycles of EE2 removal clearly show that immobilization enhanced reusability of the immobilized laccase. However, further work to reduce enzyme leaching and research in the field of biocatalyst reusability is still required because the possibility of multiple use of immobilized enzymes is their greatest advantage over free enzymes as it reduces the total costs associated with biocatalyst delivery, and improves the biocatalytic productivity of the enzyme.

Comparison of the relative activity of native and immobilized enzyme over storage time was also the key element of the research (Fig. 3b). After 5 days of storage, native and immobilized laccase retained 80% and 90% of its relative activity, respectively, whereas after 20 days it was 30% and 40%, respectively. The data show that laccase immobilized onto CaSiO$_3$ support retains higher activity than the native enzyme, due to the protective effect of the support material. Wang et al. [69,71] studied the storage stability of laccase immobilized onto zeolite imidazolate framework-67 and found that around 90% of initial relative activity was retained after 5 days of storage. The results obtained by Patil and Yadav [47] are in agreement with the presented conclusions. In the study, laccase was encapsulated into ZIF-8 support and retained about 50% of its initial activity after 7 catalytic cycles and 80% activity after 20 days of storage. By contrast native enzyme lost more than 50% of its activity at these conditions. This fact justifies the enzyme immobilization that may minimize the adverse effect of process conditions and therefore immobilized laccase retained higher activity over storage, as compared to the free biomolecule.

3.2.2. Effect of time on EE2 removal efficiency

The duration of removal process is important in terms of its efficiency and practical applicability of the proposed biosystem for degradation of estrogens. In order to confirm simultaneous mechanism of EE2 removal by adsorption and catalytic action, the crucial step included investigations of the effect of time on degradation efficiency of EE2, using free laccase and CaSiO$_3$ with active and inactivated enzyme (Fig. 4). The removal process was conducted for 12 h. However, in the case of CaSiO$_3$ containing inactivated enzyme, the highest removal of the hormone was obtained after 3 h of reaction, reaching 30%. This result clearly showed that the adsorption process itself is insufficient to remove EE2 effectively. This result was compared to the removal efficiency using pure CaSiO$_3$ as a reference sample. It was observed that the maximum extent of removal via adsorption by pure CaSiO$_3$ was 41% after 12 h of removal process. This value is higher than that obtained with the biosystem control based on CaSiO$_3$ and inactivated laccase. We ascribe the higher

Fig. 3. (a) Reusability of CaSiO$_3$-laccase biosystem with relative amount of enzyme loaded onto support and (b) storage stability of free laccase and CaSiO$_3$-laccase biosystem.

Fig. 4. EE2 removal vs. time for free laccase, CaSiO$_3$ with inactivated laccase and CaSiO$_3$-laccase biosystem.
removal efficiency obtained using pure CaSiO$_{3}$ to be a result of a higher surface area and porosity (Section 3.1.) compared to the control system comprising CaSiO$_{3}$ and inactivated laccase. Pristine CaSiO$_{3}$ appears to adsorb more EE2 molecules based on the concept that the enzyme molecules (whether inactivated or active) saturate the “active centers” in the CaSiO$_{3}$ system, and in this way decrease EE2 adsorption. However, the difference between removal efficiencies using these two types of materials is insignificant (less than 10%), therefore the focus was solely on a comparison of the removal efficiency of the CaSiO$_{3}$-laccase biosystems versus CaSiO$_{3}$ with inactivated laccase. After 3 h of the process, the CaSiO$_{3}$-laccase biosystem allowed to fully remove EE2 from aqueous solutions, whereas during the same time the free laccase removed around 70% of estrogen.

There was a noticeable increase in the final removal efficiency using immobilized laccase as compared to the free laccase and system with inactivated enzyme. This high EE2 removal efficiency is likely due to a partial stabilization of the enzyme structure after immobilization [28]. The activity retention of immobilized laccase was thus more than 85%. The obtained 100% EE2 removal rate is thus a result of the synergistic removal of estrogen by enzymatic action and sorption of pollutant by the CaSiO$_{3}$ support, with the enzymatic conversion predominating the removal efficiency. The obtained results can be compared with data presented by Lloret et al. [32]. They used a continuous enzymatic membrane reactor for removal of different estrogens with laccase from Myceliophthora thermophila as a biocatalyst. Although high amounts of biomolecules were retained on the ultrafiltration membrane in the reactor, and the removal efficiency of EE2 equaled almost 94%, the process time was 100 h with a slow removal rate of only 0.18 ng/L-h.

3.2.3. Effect of various process conditions on EE2 removal efficiency

The parameters of wastewater, such as pH, temperature or their composition depend on their source and conditions of their outlet from industry. As it was presented, the pH of wastewater from the pharmaceutical industry may equal between 3.7 to even 8.5 [72], whereas the temperature of such effluents may reach 80 °C [30]. These data was the motivation behind conducting the EE2 removal experiments under various conditions (Fig. 5). It was observed that the removal efficiency of EE2 increased with increasing pH and reached 100% at pH 5 using free and immobilized laccase (Fig. 5a). However, at pH above 5 (precisely at pH 8) the final removal rate gradually decreased and reached 9%, 23% and 32% using free laccase, CaSiO$_{3}$ with inactivated biomolecule and the CaSiO$_{3}$-laccase biosystem, respectively. The removal efficiency of estrogen by adsorption on the support with an inactivated enzyme, in the tested pH range, remained almost unchanged and fluctuated around 30%. This fact confirms that pH does not affect adsorption efficiency of EE2 onto CaSiO$_{3}$ significantly, however an effective enzyme action is hampered at basic pH, mainly due to binding of hydroxyl ions to the laccase active site. Nevertheless, within the pH range 3–7 over 60% of EE2 could be removed suggesting improved stability of the immobilized enzyme and better pH tolerance as compared to the free enzyme.

Regarding the effect of temperature on removal efficiency of EE2, 100% removal of estrogen by free laccase and its immobilized form, at 25 °C, was observed (Fig. 5b). The slight increase of EE2 removal efficiency by adsorption onto CaSiO$_{3}$ with increasing temperature, which may be due to the endothermic nature of the adsorption process, was also an interesting result [67]. However, at higher temperatures laccase...
might be partially inactivated, resulting in a decreased removal rate of EE2. The results showing the highest removal efficiency of EE2 at pH 5 and mild temperature (25 °C) are directly related to the fact that laccase possesses the highest catalytic activity at these conditions [23, 62, 63, 64].

The next investigated parameter affecting removal of EE2 was the initial concentration of pollutants. The concentration of EE2 in influents from municipal wastewater treatment plants can reach 7890 ng/L [65]. Therefore, in order to study the removal efficiencies it was decided to use EE2 in aqueous solutions at concentrations from 0.1 to 5 mg/L. Fig. 5c shows that the highest removal efficiencies were obtained for 0.1 and 1 mg/L of EE2 solutions and reached 100% for both, native and immobilized laccase. Above these EE2 levels the removal efficiency of pollutants is obtained. The possible explanation of this phenomena is the fact that higher mass of CaSiO3-based system leads to agglomeration of the particles of support material, resulting in decreasing sorption and making it impossible to reach the highest removal efficiency of estrogen by increasing amount of CaSiO3 material [29].

In summary, the highest EE2 removal efficiency of 100%, was noted when using 100 mg of the CaSiO3-laccase system at pH 5, 25 °C, at a hormone concentration of 0.1 mg/L. The results were compared with EE2 removal efficiencies obtained using free laccase and CaSiO3 with inactivated enzyme. It was shown that CaSiO3-laccase biosystem was able to remove EE2 more effectively than the CaSiO3 with inactivated enzyme and the free biomolecule over wide pH range, temperature, and estrogen concentration. It was also shown that using produced biosystem, the synergistic removal of EE2 occurred due to simultaneous adsorption of hormone molecules onto support material and their enzymatic bioconversion using immobilized laccase. These results are promising in terms of future practical application for efficient synergistic removal of pollutants from aqueous solutions.

### 3.2.4. Effect of cations and anions on EE2 removal efficiency

Cations and anions species are one of the most common water pollutants [1, 4, 44, 69, 71]. They can be discharged into drinking and surface waters from wastewaters, raising from various industries such as automobile, metallurgic, etc. The extensive industrialization causes that cations and anions are observed in various waters, such as city canals and rivers in Brazil [40], wastewater from a dry tropical area of India [58] and many other water reservoirs [15, 16, 1, 45, 59, 82]. Therefore, it was decided to investigate the effect of selected competitive cations on removal of EE2 using free laccase, CaSiO3 with inactivated laccase and CaSiO3-laccase biosystem (Fig. 6).

Decreasing removal efficiency of EE2 using all tested systems, in the presence of each of the tested cation, was observed. For instance, the removal efficiency of EE2 in the presence of Pb2+, in estrogen solution, decreased from 100% to 43% after using free laccase, whereas after application of CaSiO3-laccase biosystem it dropped from 100% to 67%. The decrease in removal rate of free laccase and CaSiO3-laccase system is probably caused by inhibition of laccase activity by selected cations in the structure of the enzyme [83]. However, free enzyme was more susceptible to decrease of its activity than the immobilized one – this proves protective effect of CaSiO3 support material onto adsorbed biomolecule [79]. Similarly, metal ions had a significant influence on the efficiency of EE2 adsorption by CaSiO3. The greatest difference between removal efficiency of EE2 by adsorption onto CaSiO3 with inactivated laccase was observed without and with the presence of Zn2+ ions - the removal efficiency of estrogen decreased from 29% to 10%, respectively. In this case, it was possible to block the active sites of the sorbent by divalent ions, which mean that EE2 molecules had to compete with them and finally the adsorption efficiency of the pollutant decreased, particularly at pH 5 (Murugesan et al., 2009). Further, the activity of the immobilized laccase in the presence of nickel ions is lower as compared to free enzyme. This might be explained by the changes of the conformation of the active site of the enzyme upon immobilization and by the fact that this changes affect enzyme properties in the presence of inhibitors/pollutants. Hence, lower activity of the immobilized biocatalyst may be related to the blocking of
presence of calcium, magnesium, sodium and potassium ions in the presented tests, leading to lower removal rate. It can be seen that the ions obtained using free laccase and CaSiO$_3$ ions using free laccase, CaSiO$_3$-inactivated laccase and CaSiO$_3$-laccase biosystems, respectively. Recently published studies have shown that these ions do not have a prominent effect on enzymatic conversion [70, 83]. In some cases, however, these ions may even suppress laccase activity. Based on the presented results, it is speculated that competition between the Ca$^{2+}$, Mg$^{2+}$, Na$^+$ and K$^+$ ions and the Cu$^{2+}$ ions in the active site of laccase occurred, leading to the reversal of the conformation of the laccase active center and changing of its activity [35]. Therefore, the removal efficiencies of EE2 in the presence of Ca$^{2+}$, Mg$^{2+}$, Na$^+$ and K$^+$ ions obtained using free laccase and CaSiO$_3$-laccase were lower as compared to the reference sample, without cations addition.

The decreasing trend in removal efficiency of EE2 was also observed in the presence of selected anions in the tested samples (Fig. 7). However, in some cases, decrease of EE2 removal efficiency is slight, especially in the presence of SO$_4^{2-}$, Cl$^-$ and Br$^-$. For instance, the removal efficiency of EE2 in the presence of SO$_4^{2-}$ remained at the same level after using free laccase, as compared to the reference sample without anions, whereas after application of CaSiO$_3$-laccase system the removal of estrogen dropped from 100% to 95%. These results stay in agreement with the study presented by Zhou et al. [83]. They showed that SO$_4^{2-}$, even at high concentration, does not affect activity of laccase, which is strictly related to effective conversion of the substrate. Interesting results were shown for removal of hormone with the presence of F$^-$ and CN$^-$. The removal efficiency of EE2 in the presence of F$^-$ and CN$^-$ equaled 54% and 32%, respectively, and be related to the synergistic effect of hormone bioconversion and adsorption onto CaSiO$_3$-laccase biocatalyst. Moreover, these drastic decrease of estrogen removal efficiency, compared to other ions, is related to the strong inhibition of laccase by fluoride and cyanide ions [60]. As shown by Koudelka and Ettinger [26], fluoride ions can decrease concentration of oxidized copper type 1 and 2 and may increase the concentration of oxygen radical intermediate. In case of the effect of CN$^-$ ions on laccase activity, it is likely that they compete with oxygen for the copper in the active site of laccase [60].

### 3.2.5. Toxicity and estrogenic activity

Significant results of the experiments are presented in Fig. 8, which shows data on toxicity and estrogenic activity of EE2 solutions, before and after treatment by free laccase, CaSiO$_3$-inactivated laccase or CaSiO$_3$-laccase biosystem. These two parameters are important in terms of possible negative effect of products of enzymatic conversion on living organisms, that affect also whole ecosystems [77]. The toxicity of solutions was measured based on mortality of *Artemia salina* organism [55], whereas estrogenic activity methodology was based on the use of β-galactosidase enzyme, the reporter gene lac-Z, which is controlled by plasmids carrying estrogen-responsive sequences [54]. Before treatment, the EE2 solution induced 100% mortality of *Artemia salina* and 100% relative estrogenic activity indicating harmfulness of the estrogen solution. After treatment of the estrogen solution by each of the systems tested, decreased mortality of *Artemia salina* and lower relative estrogenic activity was observed. After treatment of the EE2 solution with free laccase, the mortality of *Artemia salina* and relative estrogenic activity were 70% and 52%, respectively. This drop may be a result of the fact that after enzymatic conversion new products were formed, which possessed lower toxicity and estrogenic activity, as compared to the initial estrogen [22,5]. What is more, a significant drop of the analyzed parameters was noticed after using the CaSiO$_3$-inactivated laccase and CaSiO$_3$-laccase biosystem. In this case, the values of mortality of *Artemia salina* and relative estrogenic activity did not exceed 50%. The data indicate that most of the estrogen molecules were adsorbed onto the CaSiO$_3$ support and/or converted by the immobilized laccase to less toxic products. Note however, that the lowest values of toxicity and relative estrogenic activity equaled 36% and 45%, respectively, were observed after using biosystem with immobilized oxidoreductase.

In order to investigate the composition of the post-treatment mixture in more depth, attempts to follow the products of enzymatic conversion using HPLC-MS analysis, were made (Fig. 1S in Supplementary Material). Although the chromatogram of a mixture before treatment shows a clear signal for EE2, in the chromatogram after 24 h treatment this signal is not observed confirming complete removal of EE2. Note however, that on the final chromatogram there is also lack of other signals.
characteristic for possible products of laccase-catalyzed conversion, including dimers or trimers of the initial estrogen [7]. This finding, supported by the significant reduction of toxicity and estrogenic activity, suggests at least partial formation of low molecular inorganic compounds, such as CO₂ and water - the final products of laccase treatment, as reported earlier [33]. Nevertheless, partial formation of oligomers of EE2 should not be excluded. The lack of signals characteristic for these compounds could be explained by the rapid adsorption of these compounds onto the surface of the CaSiO₃ material, which was confirmed by the presence of small amount of slightly brown precipitate found on the surface of support material after the treatment process. It should be underlined, that regardless of the type of products formed, all of them are characterized by being of less harmful character than EE2, which results from decreased toxicity and estrogenic activity of the final mixture [49].

Although the advanced investigation of the products of biocatalytic conversion was not the main goal of the study, obtained data confirmed the possible dual mechanisms of enzymatic action. Nevertheless, presented results indicated that raising products of EE2 removal are less toxic than the parent compound and highlight the role of simultaneous adsorption and also possibility to adsorb both, parent EE2 and products of enzymatic conversion [46]. These results allow us to conclude that the proposed method of EE2 removal by synergistic enzymatic conversion and adsorption can be effective not only considering efficiency but also in terms of low toxicity and low estrogenic activity of post-treatment solution.

3.2.6. EE2 removal from real wastewaters

Besides the detailed characteristics of the produced systems and confirmation of their possible application for removal of EE2 from a model solution, it is crucial to assess the applicability of a given technology using real wastewater at environmentally relevant concentrations. Hence, free laccase, the CaSiO₃ system with inactivated enzyme and the CaSiO₃-laccase system were applied for removal of EE2 from two types of wastewater: (i) wastewater entering the treatment plant before treatment and (ii) wastewater leaving treatment plant after treatment (Fig. 9). It should be highlighted that the composition of the wastewater samples and EE2 concentration varies significantly depending on the type of wastewater and sampling place, affecting the removal process. In the influent sample EE2 concentration reached 3295 ng/L, whereas the level in the effluent sample was 116 ng/L. Lower concentration of the EE2 in the effluent sample as well as its less complex composition facilitated higher removal rate of estrogen by all tested systems, as compared to EE2 removal rate from sample before treatment. In fact, the biocatalytic system removed over 40% of the EE2 after 12 h from the wastewater leaving the treatment plant. Similar observations were made for the samples after treatment by laccase only or by the CaSiO₃ system with inactivated enzyme. In general, the removal efficiencies achieved for the real wastewater samples were around 60–70% lower than the efficiencies obtained for model solutions of EE2 used in this study.

This clearly shows that complexity of the wastewater solution and the presence of numerous inhibitors strongly limited enzymatic action and decreased removal rate. Moreover, the low EE2 environmental concentration made the molecules less accessible for the biomolecule additionally lowering final removal rate. Similar observations were also presented by Nguyen et al. [43], who observed more than 40% lower removal efficiency of various micropollutants, including estrogens and pharmaceuticals, in packed-bed enzyme reactor with laccase immobilized onto activated carbon, when the process was performed using real wastewater solution, as compared to the model solution.

In summary, the tests with model solutions showed very high removal rates of EE2, but the application tests using real wastewater clearly proved the potency of the CaSiO₃-laccase biosystem for removal of EE2 from real wastewater. However, further extensive studies in this area are still warranted to better understand the detailed kinetics of the removal of EE2 from different types of wastewater, including the influence of the various reaction parameters, including e.g. present cations and anions, and other components that may be found in wastewater.

4. Conclusions

In this work, CaSiO₃ was used as a support for laccase immobilization and then applied for removal of 17α-ethylxylestradiol from aqueous solution. The removal efficiencies of estrogen using the CaSiO₃-laccase biosystem were compared to free laccase and CaSiO₃-inactivated laccase system in order to select the optimum process conditions and to determine the mechanism of estrogen removal. The highest EE2 removal efficiency was observed when using the CaSiO₃-laccase biosystem, due to the synergistic removal of estrogen by adsorption onto the support material and bioconversion by laccase. At the optimal process conditions (12 h, pH 5, 25 °C, 0.1 mg/L estrogen solution, 100 mg of biocatalyst) it was possible to fully remove EE2 from aqueous solution. Due to the possible presence of various ions species in real wastewater, the effect of specific competitive ions on removal efficiency of EE2 was examined. Presence of specific cations and ions in the estrogen solution decreased the estrogen removal efficiency mainly due to a decrease of laccase activity and saturation of support active sites by the cations. Reusability and storage stability studies showed that it was possible to remove around 70% of EE2 after 5 consecutive catalytic cycles whereas immobilized laccase retained around 40% of its activity after 20 days of storage. Free and immobilized enzyme showed comparable results of removal efficiency in a single reaction cycle, yet, retention of high activity over repeated use should be of particular interest, particularly
taking into account enzyme leaching due to weak enzyme-support interactions. This factor makes immobilized enzymes more promising for practical application and determines the attractiveness of the immobilization process. The study also showed over 60% reduction of toxicity and estrogenic activity of the EE2 solution after treatment, confirming formation of less toxic products additionally followed by adsorption of estrogen molecules and products of enzymatic conversion onto the CaSiO3 support material. Our study implies that the proposed biosystem made of CaSiO3 and laccase could find application in removal of various phenolic compounds from aqueous solution by catalytic conversion supported by simultaneous adsorption onto the CaSiO3 material. Further studies are required on application of oxide systems for laccase immobilization and design of novel biocatalytic systems dedicated for removal of estrogens, pharmaceuticals, dyes or phenols from wastewater. Nevertheless, it should be noted that compared to other methods of pollutant removal (Table 1), the developed biocatalytic system is relatively easy to prepare and use, and its application is possible under mild process conditions. Despite the fact that other techniques allow for high efficiencies of the EE2 removal process, they often require use of toxic reagents, complicated equipment, or harsh process conditions. Taken together, the data obtained suggest that the biocatalytic CaSiO3-laccase system provides a prospective alternative solution.

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CRediT authorship contribution statement
Jakub Zdarta: Conceptualization, Methodology, Investigation, Manuscript writing. Formal analysis, Project supervision. Filip Ciesieleczyk: Conceptualization, Methodology, Manuscript writing. Katarzyna Jankowska: Conceptualization, Methodology, Investigation, Manuscript writing. Agnieszka Rybarczyk: Methodology, Investigation, Manuscript writing. Oliwia Dęgórka: Methodology, Investigation, Manuscript writing. Teofil Jesionowski: Project supervision, Verification of the final version of manuscript. Anne S. Meyer: Project supervision, Verification of the final version of manuscript.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability
Data will be made available on request.

Appendix A. Supporting information
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2023.109443.

References

Table 1
<table>
<thead>
<tr>
<th>Degraded compound</th>
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<th>Removal conditions</th>
<th>Removal efficiency</th>
<th>References</th>
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<tr>
<td>EE2</td>
<td>Enzymatic conversion supported by adsorption</td>
<td>12 h, pH 5 25 °C</td>
<td>100%</td>
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<td>EE2</td>
<td>Fenton-like oxidation mechanism</td>
<td>60 min</td>
<td>96.4%</td>
<td>[31]</td>
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<td>EE2</td>
<td>Use of microalgae</td>
<td>7 days microalgae cultures C. vulgaris</td>
<td>83%</td>
<td>[75]</td>
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<td>Membrane separation</td>
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<tr>
<td>EE2</td>
<td>Advanced Oxidation Processes</td>
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