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ENVIRONMENTAL BIOTECHNOLOGY

Advanced enzymatic elimination of phenolic contaminants in wastewater: a nano approach at field scale

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Abstract The removal of recalcitrant chemicals in wastewater treatment systems is an increasingly relevant issue in industrialized countries. The elimination of persistent xenobiotics such as endocrine-disrupting chemicals (EDCs) emitted by municipal and industrial sewage treatment plants remains an unsolved challenge. The existing efficacious physicochemical methods, such as advanced oxidation processes, are resource-intensive technologies. In this work, we investigated the possibility to remove phenolic EDCs [i.e., bisphenol A (BPA)] by means of a less energy and chemical consuming technology. To that end, cheap and resistant oxidative enzymes, i.e., laccases, were immobilized onto silica nanoparticles. The resulting nanobiocatalyst produced at kilogram scale was demonstrated to possess a broad substrate spectrum regarding the degradation of recalcitrant pollutants. This nanobiocatalyst was applied in a membrane reactor at technical scale for tertiary wastewater treatment. The system efficiently removed BPA and the results of long-term field tests illustrated the potential of fumed silica nanoparticles/laccase composites for advanced biological wastewater treatment.

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Introduction

The presence of emerging organic contaminants (EOCs), such as pharmaceuticals and other hormonally active chemicals in wastewater treatment plant (WWTP) effluents, in surface waters, and in groundwater, has become a matter of growing concern over the last few decades because of their potential adverse effects on human health and aquatic ecosystems (Cirja et al. 2008; Kuster et al. 2008; Zhang et al. 2011). The source, occurrence, and environmental fate of EOCs in both surface and wastewaters have been in the focus of a large number of studies during the last few decades (reviewed by Pal et al. 2010). Endocrine-disrupting compounds (EDCs) belonging to the group of EOCs are defined as "exogenous substances or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (Greim 2004) and have been demonstrated to cause various detrimental health effects that have been reported in recent years. Therefore, the occurrence and fate of EDCs in the environment, such as bisphenol A (BPA), a high production–volume chemical (Galliker et al. 2010), and building block for the production of flame-retardants, polycarbonate plastics, and epoxy resins (Staples et al. 1998), gained increasing interest worldwide. One major source of pollution is suspected to be the effluents of WWTPs (Auriol et al. 2006; González et al. 2007; Sánchez-Avila et al. 2009; Stasinakis et al. 2008; Ying et al. 2009). Alongside the ban of selected compounds and source control the improvement of WWTPs by tertiary treatments seems to

be an appropriate measure. Many advanced technologies for the elimination of micro-pollutants in wastewater treatment systems are currently investigated, i.e., oxidation, filtration, adsorption, and biodegradation (Corvini and Shahgaldian 2010).

A large number of phenolic wastewater contaminants are known to interfere with human and animal hormonal systems. For instance, BPA that, in addition to its endocrine disrupting activity is also suspected to have carcinogenic effects (Hengstler et al. 2011; Weber Lozada and Keri 2011), is found at potentially harmful concentrations (5–200 ng L^{-1}) in surfacewater, groundwater and seawater.

Development and implementation of economically and environmentally efficient techniques for BPA elimination in wastewater treatment processes remains a challenge of high concern for the society. One promising approach is based on the application of immobilized biocatalysts able to transform BPA into removable nontoxic products. This approach has been recently reported (Cabana et al. 2007; Galliker et al. 2010; Majeau et al. 2010; Mohapatra et al. 2010a). In this context, laccases are attracting an increasing interest since these metallo-enzymes are able to oxidize, polymerize, and transform a broad range of phenolics including anthropogenic compounds (Majeau et al. 2010). Furthermore, the immobilization of laccases on fumed silica nanoparticles (fsNP) has been reported to increase the enzyme stability compared to its soluble counterparts and to preserve BPA transformation activity in wastewater even at environmentally relevant conditions (Hommes et al. 2012).

There are many reports on different aspects of tertiary wastewater treatment costs (investment or operational costs) but very few publications focus specifically on EOC removal. Furthermore, comparison of different studies is impeded by different assumptions on which cost analyses are based. Differences arise on aspects related to equipment amortization costs, raw materials costs, energy costs, and labor costs (Cañizares et al. 2009). This led to huge differences in estimated costs associated with, e.g., Fenton treatment (0.2–17.7 € m^{-3}; Cañizares et al. 2009). The same trend is observed for other advanced oxidation processes such as electrochemical oxidation or ozonation (Cañizares et al. 2009). Thus, lack of information on accurate cost estimation complicates comparison and, consequently, the choice of the best available technology to eliminate EOC from domestic wastewater.

The three main objectives of this study were to demonstrate: 1) the production and applicability of one nanocomposite, i.e., laccase immobilized on fsNP, 2) the technical feasibility of a system specially designed to eliminate phenolic contaminants from wastewater and its implementation in a WWTP, and 3) to compare this novel biological process to other proposed tertiary wastewater treatment processes regarding both the results from its operation and the estimated treatment costs.

Material and methods

Chemical reagents, instruments and laccase origin

fsNP (surface area: 390±40 m^2 g^{-1}; aggregates of particles with an average size of 7 nm) from Sigma-Aldrich (Switzerland) were used as immobilization support material. 3-Aminopropyltriethoxysilane (APTES) and glutaraldehyde were purchased from Sigma-Aldrich. A plate reader (Synergy™ 2 Multi-Mode Microplate Reader, BioTek Instruments, Switzerland) and Gen5 1.08 Data Analysis Software (BioTek Instruments) were used for colorimetric assays.

Analytical grade chemicals were purchased from Fluka (Switzerland) and analytical grade solvents from J.T. Baker (Switzerland) and used without further purification. Two different buffer systems were used: Sörensen phosphate buffer (PB; 30 mM NaH_2PO_4, 40 mM Na_2HPO_4; pH 7) and McIlvaine phosphate-citrate buffer (McIlvaine 1921). Pure water (resistivity>18 MΩ cm) was obtained using a Purelab® (Elga, France) water purification system.

Laccase of a *Thielavia* genus was obtained from AB Enzymes (Germany) and precipitated with acetone (up to 80 %, on ice) before applying to an experiment in order to remove possible impurities. After centrifugation (2,500×*g*; 5 min; 4 °C) the supernatant was removed and the precipitated protein fraction was dissolved in PB buffer. The laccase was applied with a specific activity of 9±2 U mg^{-1} protein as previously determined (Hommes et al. 2012) and had an optimal activity working range between pH 5.5–6.5 according to the producer.

Assays and particle characterization

In order to determine the laccase activity colorimetric 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) oxidation tests were carried out as previously described (Zimmermann et al. 2011). One unit was defined as the amount of laccase oxidizing 1 μmol ABTS min^{-1}. Protein contents were determined according to Lowry et al. (1951). Bovine serum albumin (BSA) was used for calibration and subsequent quantification.

The efficiency of aminopropylation was determined following a ninhydrin assay described elsewhere (Sun et al. 2006). Furthermore, changes of the surface charge due to functionalization of the fsNP were determined by means of zeta potential measurement as described before (Galliker et al. 2010). Additionally, mass increases of fsNP after aminopropylation and after immobilization of the enzymes were measured by means of thermo gravimetrical analysis using a Thermobalance (Mettler TC 15 TA controller equipped with a Mettler TG 50). An aliquot of 10 mg lyophilized fsNPs were heated from 50 to 700 °C at a heating rate of 10 °C min^{-1} under aerobic environment.

Rheological measurements

Rheological responses of particle suspensions of different particle densities and with different surface modifications were determined using a plate-and-plate geometry (plate diameter 40 mm and plate-to-plate gap 0.15 mm) on a Bohlin Gemini advanced rheometer (Malvern Instruments, UK). The particle suspensions were directly applied to the plate and experiments were conducted at a constant temperature of 40 °C. The data was fitted with the Ostwald–de Waele relationship (Ostwald 1925):

$$\tau = K\left(\frac{\partial u}{\partial y}\right)^{n}$$

with τ being the dynamic viscosity, K the flow consistency index, i.e., the dynamic viscosity at a shear rate of 1 s^{-1}, $\partial u/\partial y$ the shear rate or the velocity gradient perpendicular to the plane of shear in s^{-1}, and n the flow behavior index.

Enzyme immobilization

Immobilization of laccase was carried out according to the general principle described in previous publications (Hommes et al. 2012; Zimmermann et al. 2011). A suspension of 100 g fsNP L^{-1} was prepared using PB at pH 7. The suspension was homogenized with a magnetic stirrer (RCT basic IKAMAG® or RET basic IKAMAG®, IKA®, Germany) at 700 rpm and 0.8 mmol APTES g^{-1} fsNP was added during stirring. The thixotropic sample was shaken until it became liquid and placed on an overhead shaker (Reax 2, Heidolph, Germany) for 24 h at 20 °C. The excess APTES was washed away by two successive centrifugation/resuspension steps [2,900×g for 15 min on a MSE Mistral 3000E centrifuge (MSE, England)]. 1 kU of laccase g^{-1} fsNP was added before the suspension was placed on an overhead shaker and treated for 2 h at 4 °C. Afterwards, the suspension was stirred at 1,200 rpm and 1.0 mmol glutaraldehyde g^{-1} fsNP was added dropwise. The suspension was again treated on an overhead shaker for 24 h at 4 °C. Subsequently, the excess enzymes and glutaraldehyde were washed away by two centrifugation/resuspension steps at 2,900×g for 15 min.

Elimination of BPA in wastewater using Lac-fsNP in pilot-scale experiments

Wastewater effluent

Removal of BPA by laccase immobilized on fsNP (Lac-fsNP) from treated WWTP effluent was investigated in a pilot-scale experiment. The experiment was conducted at the WWTP Birs located in Birsfelden, Switzerland. The plant is treating both domestic and industrial wastewater from the surrounding area with a design capacity of 150,000 population equivalent (PE). The treatment train in WWTP Birs is made up of mechanical pretreatment (grit chamber, coarse screen, sand trap, and fine screen) followed by activated sludge treatment. The effluent from the sand trap is pumped directly to the activated sludge reactor without primary settling. The activated sludge treatment consists of five parallel tanks (8,100 m^3 each) operated as sequencing batch reactors. The operational sequence of each reactor includes filling–denitrification–nitrification–settling–decanting. During the decanting phase 20–30 % of the clear water on the top of the tank is discharged. The excess sludge is partly dewatered in a centrifuge and pumped to a digester for further treatment. The WWTP removes, on average, 97 % of biochemical oxygen demand, 85 % of total nitrogen, and 86 % of total phosphorus from the raw wastewater. Parameters of the treated wastewater effluent from WWTP Birs were measured during the experimental period, i.e., from December 2010 to March 2011. Average temperature was 13.2±1.5 °C, while average concentrations of ammonium nitrogen, total nitrogen, total phosphorus, dissolved organic carbon, and total suspended solids were 0.5±0.2, 6.2±2.2, 0.7±0.2, 7.8±1.8, and 6.9±4.7 mg L^{-1}, respectively. Chemical oxygen demand was, on average, 30.1±2.9 mg O_2 L^{-1} and pH was, on average, 8.7±0.7. All these values are common for treated wastewater effluent from WWTP Birs.

Membrane reactor—process design and operational parameters

A membrane reactor with a 460 L effective reactor volume (MMS, Switzerland) was used in pilot-scale experiments. The process scheme is depicted in Fig. 1. An ultra-filtration (UF) membrane module (BIO-CEL®, polyether sulfone, 0.04 μm pore size, 10 m^2 membrane area, Microdyn-Nadir, Germany) was placed in the reactor. Air diffusers were installed at the bottom of the reactor and air flow per membrane area was set at 0.6 V_n m^{-2} h^{-1} in order to facilitate hydraulic mixing and prevent membrane fouling.

The pilot plant was operated continuously in a cyclic filtration mode. The cyclic filtration protocol included four sequential phases, i.e., permeate production (7.5 min), relaxation (0.5 min), membrane backwash with permeate (0.5 min), and relaxation (0.5 min). The permeate production rate was set at 100 L h^{-1} corresponding to a net permeate flow of 78 L h^{-1}. The treated effluent was fed continuously into the reactor. Foaming was prevented by dosing approximately 4 mL of anti-foaming agent (CORASIL-1818, CORAG Switzerland) per day into the reactor. The pilot plant was fully automated and parameters including flow rates and trans-membrane pressure were logged.

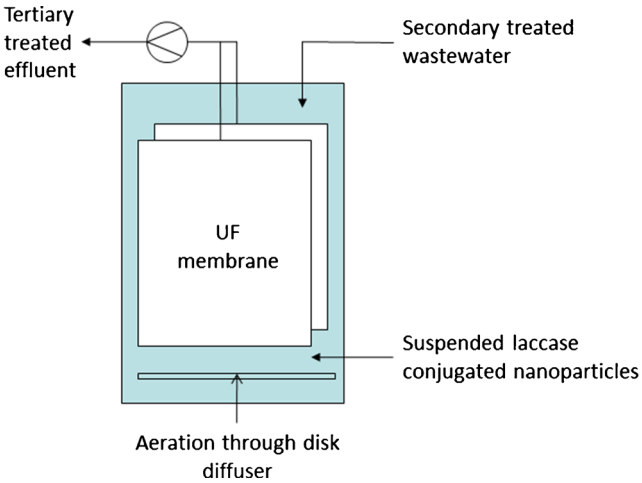

Fig. 1 Process scheme of the membrane reactor pilot plant as a tertiary treatment step

In the pilot experiment, a total of 0.5 kg of Lac-fsNP with a specific activity of 1.23 kU g^{-1} Lac-fsNP were applied at once. Therefore, a laccase activity of 1.34 kU L^{-1} with a particle load of 1.09 g Lac-fsNP L^{-1} reactor volume was applied. Treated wastewater effluent from WWTP Birs was fed continuously into the reactor at temperatures between 10 and 15 °C.

Wastewater parameters including ammonia nitrogen (NH_4-N; LCK303 HACH LANGE), nitrate nitrogen (NO_3-N; LCK339 HACH LANGE), BPA concentrations (GC-MS, Hummel et al. 2006), dissolved organic carbon (DOC; TOC-V wp, Shimadzu), pH, and temperature in both feed and permeate were measured twice per week. 24-h-flow proportional samples (composite sampling) were collected for the experiment. Furthermore, in order to monitor the residual enzymatic activity in the membrane reactor over the course of the experiment, samples were taken daily from the feed, permeate, and reactor for laccase activity measurements.

Statistical analysis has been performed in order to evaluate whether BPA concentrations in the WWTP effluent were significantly different from BPA concentrations in the permeate after treatment in the membrane reactor. The data sets were tested for normal distribution using the Shapiro−Wilk normality test (chosen significance level $p < 0.2$). The Bartlett test of homogeneity of variances was used to test whether variances are equal (chosen significance level $p < 0.2$). Analysis of variance (ANOVA) was used for normally distributed data sets with equal variances (chosen significance level $p < 0.05$). One-way ANOVA by ranks (Kruskal−Wallis) was used for data that were not normally distributed (chosen significance level $p < 0.05$).

Evaluation of economic feasibility

The treatment costs for WWTP were calculated on the basis of a daily flow of 28,000 m^3 day^{-1} (operator's data). For electricity cost, a price of 0.1 € kWh^{-1} was used for further calculations (Eurostat 2011).

Estimation of Lac-fsNP-production cost

The calculations of production cost were done taking the commercial prices of the support material and chemicals for cross-linking and surface modification into account. Energy costs were calculated for centrifugation and cooling systems. Prices for laccase differ drastically depending on the origin and availability. Therefore, the commercially available laccase from genus *Thielavia*, i.e., the laccase used in the present study was considered for calculations. Personnel costs were not taken into account.

Estimation of investment and operational costs

The investments considered for the cost calculation included two principal components of the up-scaled membrane reactor: a single reactor vessel and a membrane unit. The sizing of the reactor and membrane modules was based on the retention time and flux applied during pilot-scale UF experiments. The conversion of the single investment into capital expenditures considered yearly interest rates of 4.5 % and depreciation periods of 30 years, 12 years, and 1 year for constructions, membranes, and biocatalysts, respectively. The calculation of operating expenditures considered energy costs for filtration and aeration. Personnel costs were not taken into account.

Results

Process optimization for kilogram-scale production of nanobiocatalyst

Optimization of the applied particle concentration

In our previous studies, the production process of nanobiocatalysts regarding quantities of applied chemicals and enzymes was optimized (Hommes et al. 2012; Zimmermann et al. 2011). In the current study, the optimization was carried out to allow for large-scale production need. Based on the protocol described before (Hommes et al. 2012), the same mass ratios of coupling chemicals to nanoparticles were applied for laccase immobilization, but with increasing particle amount per reaction volume. Finding the optimal particle concentration during the immobilization procedure, i.e., the highest particle concentration at which particle suspensions could still be stirred without using excessive energy, was an essential developmental step for more cost-effective production. Therefore, the rheological characteristics of different particle suspensions (Fig. 2), which originated from the individual immobilization process steps, were investigated.

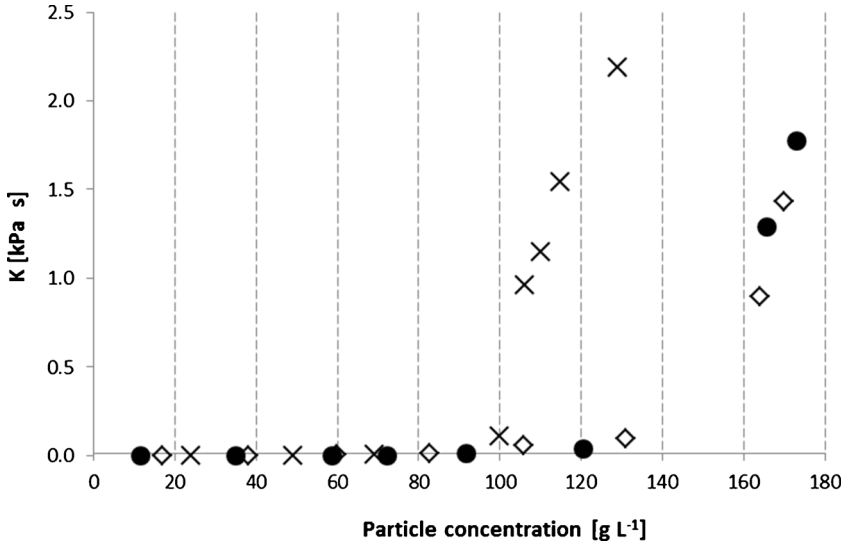

Fig. 2 Rheological responses, i.e., flow consistency indexes of fsNP (*crosses*), AP-fsNP (*diamonds*), and Lac-fsNP (*black circles*) in suspensions of different particle concentrations

With increasing particle concentration viscosity showed a rapid increase at a critical point depending on the surface modification. This critical point was reached at approximately 100 g of particles L^{-1} for fsNP. Higher concentrations led to drastically higher viscosities of the working suspensions greatly hampering treatability. Therefore, a particle concentration of 100 g fsNP L^{-1} was deemed to be optimal, and consequently, 0.08 mol APTES and 100 g fsNP L^{-1} medium were used for surface modification of the fsNP. In the subsequent coupling step, aminopropylated fsNP (AP-fsNP) were applied, which showed a slight mass increase of approximately 6 % compared to applied fsNP mass due to surface modification. The obtained amount of 106 g AP-fsNP L^{-1} was suspended and applied for laccase immobilization. Again, an increase of particle mass of approximately 15 %, due to the loading of fsNP with glutaraldehyde and protein, was measured after immobilization of the enzymes and washing of the particles. Thus, starting the production with 100 g fsNP L^{-1}, the final particle concentration was 121 g L^{-1} after the whole immobilization procedure. Particle concentration was never above the critical point and treatment was not hampered by too high viscosity of the suspension since both AP-fsNP and Lac-fsNP showed a critical increase in viscosity well after 130 g particles L^{-1}.

Monitoring of surface modification and laccase immobilization at kilogram scale

After each process step, different control measurements were performed in order to determine the efficiency of the adapted protocol. Results from amino group quantification assays and zeta-potential measurements after aminopropylation with APTES indicated successful surface modification of fsNP. The nitrogen content increased from 0.25±0.05 mg N g^{-1} fsNP for non-modified fsNP up to 16.0±0.1 mg N g^{-1} fsNP after aminopropylation and the surface charge changed drastically from −25.1±0.4 for fsNP to 6.31±0.10 for AP-fsNP. Those results confirmed the successful modification of the surface of the nanoparticles.

Monitoring enzymatic activity of the biocatalysts during the laccase immobilization procedure indicated that virtually the whole enzyme load could be conjugated to the particles. Only 3.6±9.5 % of laccase activity was lost during the washing procedure. Washing losses were determined as the relative difference of laccase activity of the suspension before (100 %) and after exhaustive washing. The residual enzymatic activity obtained after the whole production was 132.5±11.5 % of initially applied laccase activity. Thus, an enzyme load of 1.23 ±0.11 kU g^{-1} fsNP was achieved.

Removal of BPA by Lac-fsNP in tertiary wastewater treatment

BPA concentrations in the treatment train without Lac-fsNP

Before dosing the Lac-fsNP, the membrane pilot-plant was fed with treated wastewater from WWTP Birs for 2 months. BPA concentrations were monitored in the raw wastewater, treated wastewater from WWTP Birs, and the UF permeate from the pilot plant. The BPA concentrations in raw wastewater showed large variations and were strongly influenced by sampling conditions, e.g., week days or weekend as well as wet or dry weather conditions. On average, BPA was found at 1.03±0.30 μg L^{-1} in the raw wastewater. Approximately 75 % of BPA was eliminated by biological treatment of the wastewater resulting in BPA concentrations between 0.04 and 0.67 μg L^{-1} and between 0.06 and 0.32 μg L^{-1} in the treated effluent and UF permeate, respectively. ANOVA of the data showed that there were no significant differences of BPA concentrations between the WWTP effluent and the UF permeate (p =0.926). Hence, it was concluded that the membrane

reactor did not further remove BPA from the biologically treated wastewater effluent.

Lac-fsNP catalysed BPA removal in membrane reactor

Laccase activity and concentrations of BPA were determined in both WWTP effluent and UF permeate (Fig. 3a). Additionally, the laccase activity in the reactor was monitored (Fig. 3b). Based on the measured laccase activity of treated wastewater effluent (feed of the membrane reactor) and UF permeate, it seems clear that the UF membrane was capable of keeping all Lac-fsNP within the reactor since virtually no laccase activity in the wastewater effluent and the UF permeate was measured.

The whole pilot-scale experiment can be divided into three phases, i.e., phase 1 (day 1–18) in which the Lac-fsNP were applied and continuous treatment of wastewater effluent was conducted, phase 2 (day 19–30) in which the reactor was run in batch mode (aeration of the reactor was continued, but continuous treatment of wastewater effluent was suspended), and phase 3 (day 31–43) in which continuous treatment of wastewater effluent was resumed.

During phase 1 BPA concentrations in the wastewater effluent and the UF permeate were, on average, 0.34±0.09 and 0.24±0.08 μg L^{-1}, respectively. However, considerable variations in BPA concentrations occurred (Fig. 3a). Nevertheless, BPA concentrations in the UF permeate were significantly lower than BPA concentrations in the wastewater effluent (as determined by one-way ANOVA by ranks; $p < 0.001$), indicating that application of the Lac-fsNP led to BPA removal.

The laccase activity in the reactor was also subjected to considerable fluctuations during phase 1. On average, the activity was 0.59±0.30 kU L^{-1} corresponding to 44±22 % of the initially applied laccase activity. However, there was no clear decline in laccase activity. On the contrary, highest activities were measured between days 13 and 16, i.e., close to the end of phase 1. Therefore, there was no indication for losses of laccase activity over time during phase 1. During

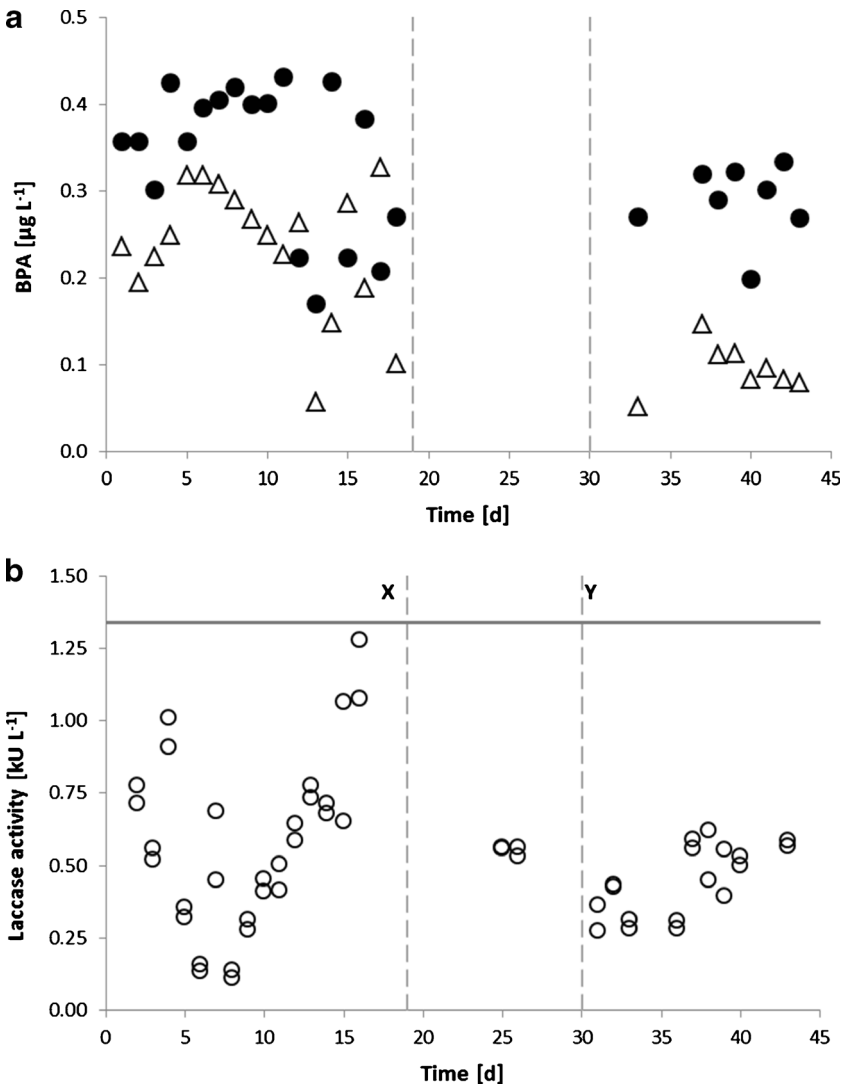

Fig. 3 BPA measured in the WWTP effluent (*black circles*) and UF permeate (*triangles*) during Lac-fsNP treatment (**a**) and laccase activity in the membrane reactor during Lac-fsNP treatment (**b**). *X* depicts the time when batch mode in the membrane reactor was started, i.e., when there was no filtration just aeration. *Y* depicts the time when continuous mode was restarted, i.e., when there was filtration and aeration again. The *dark grey line* in Fig. 3b at 1.34 kU L^{-1} depicts the initially applied laccase activity

phase 2, laccase activities were determined on days 25 and 26. They were, on average, 0.55±0.01 kU L^{-1} corresponding to 41±1 % of the initially applied laccase activity. Therefore, the Lac-fsNP seemed to remain stable during batch mode.

During phase 3, concentrations in the wastewater effluent and the UF permeate were, on average, 0.29±0.04 and 0.10± 0.03 μg L^{-1}, respectively. Variations between BPA concentrations were considerably lower than during phase 1. As during phase 1, BPA concentrations in the UF permeate were significantly lower than in the wastewater effluent (as determined by ANOVA; $p<0.001$) reinforcing the result from phase 1 that application of the Lac-fsNP improved BPA removal. Based on the difference in average BPA concentrations of the wastewater effluent and the UF permeate, it can be estimated that approximately 0.19 μg L^{-1} corresponding to 66 % of the feed BPA could be transformed due to the tertiary treatment with Lac-fsNP in the membrane reactor during phase 3.

During phase 3, laccase activities were subject to lower variations as during phase 1 as well; they were 0.44± 0.12 kU L^{-1} or 33±9 % of the initially applied laccase activity. Again, there was no clear decline in laccase activity over time. On the contrary, lowest laccase activities were rather measured at the beginning of phase 3. Hence, there was no indication for losses in laccase activity during phase 3. Over the whole experiment, Lac-fsNP seemed to retain around 30 % to 40 % of their initial enzymatic activity.

Estimated production costs and expenditures for tertiary wastewater treatment

Table 1 summarizes the estimated costs for the production of the nanobiocatalyst and Table 2 summarizes the estimated capital and operating expenditures necessary for tertiary wastewater treatment with Lac-fsNP. Total capital expenditures and total operating expenditures amount to 0.066 € m^{-3} and 0.064 € m^{-3}, respectively. This corresponds to total costs of 0.130 € m^{-3} or to daily costs of 0.021 € PE^{-1}, assuming a wastewater production of 160 L PE^{-1} (as assumed by Rosenstiel and Ort 2008).

Discussion

Kilogram-scale production of Lac-fsNP

Immobilization of laccases on solid surfaces is of increasing interest due to the broad range of possible applications for these enzymes and the considerable improvements in biocatalytic process economics associated with enzyme immobilization due to improved enzyme stability, facilitated reuse, and easier separation from product (Brady and Jordaan 2009). Several research groups have successfully immobilized laccases on different support materials in lab scale experiments (e.g., Mohidem and Mat 2009; Qiu et al. 2009; Rekuć et al. 2009; Zhu et al. 2007). In the present study, an already developed and optimized method for laccase immobilization on lab scale (Hommes et al. 2012; Zimmermann et al. 2011) has been successfully adapted for the production of Lac-fsNP on the kilogram scale.

The crucial improvement for faster and more efficient production was the increase of particle concentration during the immobilization procedure. Results of rheological measurements showed that fsNP concentrations above 100 g L^{-1} led to a steep increase in viscosity of the particle suspension, thereby severely hampering further processing. Therefore, an fsNP concentration of 100 g L^{-1} was applied. Laccase immobilization resulted in particles with a laccase activity of 1.23±0.11 kU g^{-1} fsNP corresponding to 132.5±11.5 % of the initially applied laccase activity. An increase in enzymatic activity due to immobilization was expected since previous lab-scale experiments showed that laccase from genus *Thielavia*, i.e., the laccase used in the present study, has increased enzymatic activity towards ABTS after cross-linking with glutaraldehyde (Hommes et al. 2012). This is also in accordance with other studies reporting increased laccase activity due to cross-linking (Durán et al. 2002) or immobilization (e.g., Mohidem and Mat 2009).

Almost the same amount of laccase activity could be immobilized using higher particle concentrations compared to particles produced on lab scale which had an activity of

Table 1 Estimated production cost of the novel nanobiocatalyst at kilogram scale

Parameter	Estimations and calculation	Reference
Biocatalyst production expenditures		
fsNP modification and laccase coupling		
Chemicals, support material, etc.	30 € kg^{-1} fsNP	(Hommes et al. 2012)
Production yield—particle per volume	100 kg m^{-3}	Present publication
Energy consumption (total)	0.198 kWh kg^{-1}	measured data;
	2.2 kWh m^{-3} [a]	Molina Grima et al. (2003)
Total production expenditures (BPEX)	30.3 € kg^{-1} Lac-fsNP [b]	

[a] Due to volume exchange for washing, a total of 0.09 m^3 kg^{-1} fsNP of PB was required

[b] Personal costs and expenditures for equipment not considered

Table 2 Estimated expenditures for tertiary wastewater treatment using the Lac-fsNP within an UF reactor

Parameter	Estimations and calculation	Reference
Capital expenditures		
Membranes		
Net filtration flux	7.7 L m^{-2} h^{-1}	Present publication
Membrane area needed	151,515 m^2	
Price per m^2 membrane	35 €	Kraume and Drews (2010)
Investment	5,303,030 €	
Capital expenditures	1,592 € day^{-1}	
	0.057 € m^{-3}	
Reactor		
Volume	6970 m^3	
Tank building costs	220 € m^{-3}	Verrecht et al. (2010)
Investment	1,530,000 €	
Capital expenditures	258 € day^{-1}	
	0.009 € m^{-3}	
Total capital expenditures (CPEX)	1,850 € day^{-1}	
	0.066 € m^{-3}	
Operating expenditures		
Filtration		
TMP	0.2 Bar	Own experiment
Pump efficiency	0.6	Gander et al. (2000)
Operating expenditure	26 € day^{-1}	
	0.001 € m^{-3}	
Aeration		
Specific energy consumption	0.3 kWh m^{-3}	Kraume and Drews (2010)
Operating expenditures	840 € day^{-1}	
	0.030 € m^{-3}	
Biocatalyst		
Biocatalyst amount per reactor volume	1.6 kg m^{-3}	present publication
Investment	340,000 €	
Operating expenditures	927 € day^{-1}	
	0.033 € m^{-3}	
Total operating expenditures (OPEX)	1,793 € day^{-1}	
	0.064 € m^{-3}	
Total costs	3,643 € day^{-1}	
	0.130 € m^{-3}	

1.53±0.02 kU g^{-1} fsNP (Hommes et al. 2012). Therefore, the undertaken changes in the immobilization procedure to increase production efficiency had no considerable repercussions for the end product.

Application of Lac-fsNP in tertiary wastewater treatment

BPA removal by tertiary wastewater treatment

A pilot-scale experiment studying one possible application for the produced Lac-fsNP, i.e., tertiary wastewater treatment, was conducted. The degradation of one EOC, i.e., BPA, was studied. BPA was chosen, on the one hand, because of its occurrence in wastewater effluents in potentially environmentally harmful concentrations and, on the other hand, because of several reports showing that laccases are able to transform BPA (e.g., Cabana et al. 2009; Demarche et al. 2012; Fukuda et al. 2004; Galliker et al. 2010) even at environmentally relevant concentrations (Hommes et al. 2012).

The pilot-scale experiment using Lac-fsNP for tertiary treatment was conducted in three phases, i.e., two phases in which wastewater effluent was treated continuously (phase 1

and phase 3) and one phase in which the bioreactor was run in batch mode without inflow (phase 2). During phase 1, considerable fluctuations in BPA concentrations and laccase activities occurred. This might be attributed to in-homogeneity of the reactor medium caused by suboptimal mixing. The low laccase activities between days 5 and 10 might be attributed to particle coagulation and sedimentation. The observed higher BPA concentrations in the permeate during these days are in accordance with this finding. Remarkably, our results indicate that the difference in BPA concentrations between wastewater effluent and permeate can be ascribed to the activity of Lac-fsNP. The subsequent increase in laccase activity and corresponding decrease in BPA permeate concentrations might be attributed to better mixing (short manual mixing of the reactor in addition to the aeration) bringing the settled particles back into suspension.

Fluctuations between days 12 and 18 in BPA concentrations were due to considerably lower BPA concentrations in the wastewater effluent on days 12, 15, and 17. Due to the continuous stirred-tank reactor character of the pilot plant with a hydraulic retention time of approximately 7 h, the drop in BPA permeate concentrations was following with a delay leading to temporarily higher BPA concentrations in the permeate than in the wastewater effluent and, consequently, to lower BPA concentrations in the permeate on days 13, 16, and 18.

During phase 3, more stable conditions than during phase 1 could be established as reflected by smaller fluctuations of measured BPA concentrations and laccase activities. BPA concentrations in the wastewater effluent were, on average, 66 % lower after tertiary treatment during this phase, giving further evidence that BPA was removed to some extent by the Lac-fsNP.

Besides BPA degradation due to oxidation by laccase, there are other possible effects that might have led to a decrease in BPA concentrations in the permeate, i.e., microbial degradation and sorption of BPA on the applied particles. Microbial degradation seems to have had no substantial effect on BPA removal since no significant differences in BPA concentrations of the wastewater effluent before and after tertiary treatment without particles could be observed. Sorption of BPA on fsNP with BSA immobilized on the surface was investigated previously in the frame of lab-scale experiments investigating BPA transformation by Lac-fsNP (Hommes et al. 2012). No BPA sorption could be observed (unpublished data), indicating that sorption of BPA on Lac-fsNP might only be of minor importance. These findings indicate that the observed decrease in BPA concentration due to tertiary treatment is mainly due to BPA oxidation catalysed by the Lac-fsNP. However, in further pilot-scale experiments with WWTP effluent where BPA concentrations in the membrane reactor feed were between 12 and 63 ng L^{-1}, i.e., considerably lower compared to the present study, no further decrease of BPA concentrations due to Lac-fsNP could be observed (data not shown), indicating that enzymatic BPA transformation at such low concentrations might occur too slow or not at all. This is in accordance with the present study in which during phases 1 and 3 no BPA permeate concentrations lower than 50 ng L^{-1} were measured.

Stability of Lac-fsNP

The Lac-fsNP appeared to retain a substantial amount of their initial enzymatic activity over the whole experiment, since no clear trend towards lower laccase activities could be observed over time and 43 % of the initially applied laccase activity could still be measured after 43 days, i.e., at the last day that activity measurements were conducted. Therefore, fluctuations of the measured laccase activities were more likely influenced by mixing issues and sedimentation or coagulation of the particles (as discussed above) than by losses in enzymatic activity. This is also supported by lab experiments in which laccases immobilized on fsNP were shown to retain most of their enzymatic activity in wastewater (Hommes et al. 2012; Zimmermann et al. 2011).

Comparison of performance between Lac-fsNP and other tertiary treatment processes

Next to tertiary wastewater treatment with immobilized laccases, physico-chemical methods for EOC removal like ozonation, Fenton oxidation, or photocatalytic oxidation are usually proposed (reviewed by Mohapatra et al. 2010b). While physico-chemical treatments can be very effective for the removal of organic pollutants (BPA removal of up to 100 % has been reported using ozonation by Lee et al. 2003) and a wide range of pollutants can be transformed, they depend on the addition of catalysts and oxidants to the solution which may be both expensive and lead to secondary pollution (Hu et al. 2002; Korshin et al. 2006). Furthermore, very strong oxidants are used in treatments like ozonation or Fenton oxidation which cause the formation of various oxidation by-products that may be more toxic than the initial substrates (Mohapatra et al. 2010b).

Next to oxidative methods, removal of EOCs via adsorption on activated carbon is another treatment option considered. BPA adsorption on granular activated carbon has been reported as effective method for BPA removal (Choi et al. 2005). In comparison to oxidative methods, adsorption on activated carbon has the advantage that no undesirable side products are produced and that the mechanism of removal is unambiguous. Furthermore, the compounds are completely removed from the wastewater stream. However, addition of activated carbon is necessary during the process: sewage sludge amounts are increased, and already treated wastewater effluent is loaded again with solids making a filtration step necessary. Furthermore, compounds that have a low affinity to adsorb on activated carbon might be displaced with

substances that have a higher affinity for the sorption material leading to desorption (Rosenstiel and Ort 2008).

Laccases have a narrower substrate range compared to the physico-chemical methods mentioned above. They predominantly oxidize polyphenols, methoxysubstituted phenols, and aromatic diamines (Singh Arora and Kumar 2010). However, the addition of co-substrates is unnecessary since they use oxygen as oxidizing agent. Studies investigating transformation products after laccase treatment of, e.g., BPA or diclofenac have shown that these compounds predominantly polymerize which leads to larger compounds more prone to precipitation (Fukuda et al. 2004; Galliker et al. 2010; Hommes et al. 2013). In case of BPA transformation, smaller compounds, i.e., phenol and 4-isopropenylphenol, have been identified but occurred in substantially lower quantities than polymerization products (Galliker et al. 2010). In case of diclofenac transformation, only polymerization products were found (Hommes et al. 2013). BPA transformation products after laccase treatment have been studied with regard to their estrogenic activity in lab-scale experiments (Fukuda et al. 2004). Structural analysis of reaction products showed the formation of various BPA oligomers most likely formed through successive oxidative condensation reactions. A luciferase reporter assay showed no estrogenic activity for the soluble as well as the insoluble part of BPA reaction products (Fukuda et al. 2004) indicating that BPA transformation by laccase enzymes leads to WWTP effluents with decreased estrogenic activity.

However, improvements of the tertiary wastewater treatment with Lac-fsNP are necessary in order to reliably transform the target compounds in equal measure as the physico-chemical methods mentioned above. Measures to increase mixing in the membrane reactor pilot plant seem to be necessary in order to decrease fluctuations in laccase activity and BPA permeate concentrations. Furthermore, nanobiocatalyst load and hydraulic retention time should be optimized in order to increase BPA removal. Further improvements increasing removal rates and broadening the range of target substrates might be achieved by immobilizing additional enzymes targeting other substrates on the same carrier, as suggested by recently published lab-scale results (Ammann et al. 2013) or through the addition of redox mediators.

Costs for tertiary wastewater treatment using ozonation and powder-activated carbon (PAC) adsorption have been chosen as point of reference, even though direct comparison with the tested Lac-fsNP treatment is problematic since the treatments differ with respect to the range of targeted substrates and removal efficiency. Yearly costs for ozonation and PAC adsorption were estimated based on cost estimations provided by Rosenstiel and Ort (2008) for the WWTP Au (Switzerland) excluding personnel and maintenance costs. The WWTP Au was chosen as reference because of its similar size compared to the WWTP Birs. The WWTP Au and Birs are treating 33,000 and 28,000 m^3 wastewater per day, respectively.

Costs for ozonation and PAC were estimated to be 0.078 and 0.114 € m^{-3}, respectively. Hence, the estimated costs for the Lac-fsNP treatment are slightly higher with 0.130 € m^{-3}. However, costs of Lac-fsNP treatment could be lowered by a more cost-efficient production of the biocatalysts and/or the production of biocatalysts with more enzymatic activity per g fsNP. Lab-scale experiments have shown that production of Lac-fsNP with 2.30 kU g^{-1} could be achieved if a loss of approximately 15 % of the applied enzymes is tolerated (Hommes et al. 2012). This would allow applying a lower particle load or reducing the size of the treatment unit since lower hydraulic retention times would be needed while achieving the same removal. While production costs for Lac-fsNP would slightly increase from 30.3 to 32.8 € kg^{-1}, approximately 155,000 € in investments could be saved corresponding to 0.018 € m^{-3} by applying the Lac-fsNP with higher activity at half of the particle load used previously.

Overall, the first pilot-scale application to remove BPA from treated wastewater by means of a novel enzymatic treatment technology on pilot scale showed positive results. The applied biocatalysts retained a substantial amount of their enzymatic activity over the whole measurement period (between 30 % and 40 %) and approximately 66 % of BPA could be removed from wastewater effluents. The costs for the treatment are in the same range as for other tertiary wastewater treatment processes considered, i.e., ozonation and PAC adsorption, and could be further reduced by optimization of the treatment technology and biocatalyst production. The studied treatment technology has large potential for wastewater treatment and its optimization should be subject to further investigations. Next to improvements of the process concerning mixing conditions, nanobiocatalyst load, and hydraulic retention time with the goal to reach more steady compound removal, immobilization of additional enzymes on the same carrier should be considered in order to allow for the removal of a broader spectrum of substances.

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