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Cell Surface Display Fungal Laccase as a Renewable Biocatalyst for Degradation of Persistent Micropollutants Bisphenol A and Sulfamethoxazole

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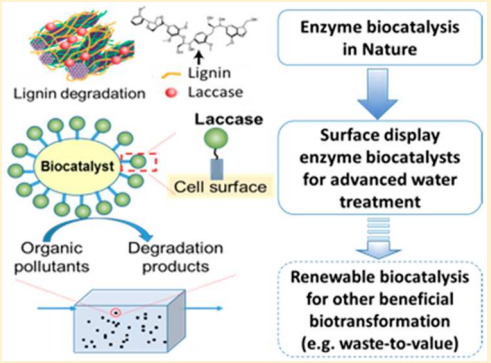
Supporting Information

ABSTRACT: Fungal laccases have high activity in degrading various persistent organic pollutants. However, using enzymes in solution for water treatment has limitations of nonreusability, short enzyme lifetimes, and high cost of single use. In this study, we developed a new type of biocatalyst by immobilizing fungal laccase on the surface of yeast cells using synthetic biology techniques. The biocatalyst, referred to as surface display laccase (SDL), had an enzyme activity of 104 ± 3 mU/g dry cell (with 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS)). The SDL retained over 90% of the initial enzyme activity after 25 days storage at room temperature, while, in contrast, activity of free laccase declined to 60% of its initial activity. The SDL could be reused with high stability as it retained 74% of initial activity after eight repeated batch reactions. Proof-of-concept evaluations of the effectiveness of SDL in treating contaminants of emerging concern were performed with bisphenol A and sulfamethoxazole. Results from contaminant degradation kinetics and the effects of redox mediator amendment provided insights into the factors affecting the efficacy of the SDL system. This study reports, for the first time, the development of a surface display enzyme biocatalyst as an effective and renewable alternative for treating recalcitrant organic micropollutants.



INTRODUCTION

Widespread detection of emerging contaminants such as pharmaceuticals and personal care products (PPCPs) has led to considerable public concern about potential risks to ecosystem and human health associated with water reclamation and reuse.[1−3] These emerging contaminants are biologically active and potentially toxic, even at trace concentrations.[4] Many emerging contaminants are endocrine-disrupting compounds (EDCs) that can cause impaired endocrine systems and developmental disorders in humans and wildlife.[5] In addition to EDCs, antibiotics are another group of emerging contaminants of serious concern. Release of antibiotic compounds to the natural environment could lead to proliferation of resistant pathogens and alteration of environmental microbial communities.[6,7] The pathway for a number of emerging contaminants entering the environment is through wastewater treatment plants, as these contaminants tend to be persistent or only partially removed by conventional wastewater treatment processes (WWTPs).[8,9] Some physical- and chemical-treatment methods, such as pressure driven membrane processes and advanced oxidation processes, have been developed for treating emerging contaminants in wastewater, especially those that pass through the conventional secondary or tertiary treatment process.[2] However, these methods face challenges for sustainable widespread implementation because of disadvantages such as high energy consumption and high costs,[10] formation of membrane concentrates enriched in contaminants that need to be further treated or disposed of,[11] or generation of byproducts with unknown or even higher toxicity.[12] Therefore, there is a critical need to develop innovative advanced persistent micropollutant treatment technologies that are effective, economical, and environmentally sustainable.

Enzyme biocatalysis can be a promising alternative to current advanced treatment processes for removing organic contaminants from wastewater streams with advantages of high activity under ambient conditions of temperature and pressure, low energy requirements, low toxicity, and simple process control and maintenance.[13,14] Laccases (EC 1.10.3.2) from the white-rot fungi of the phylum Basidiomycota have received increasing attention for their high biochemical activity in catalyzing degradation of recalcitrant organic compounds.[15] Fungal laccases were first identified as lignin degrading enzymes[16]

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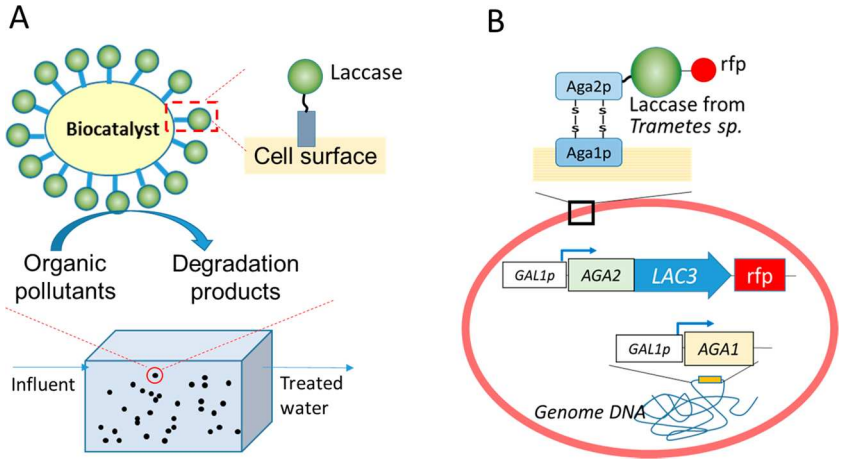

Figure 1. Overview of the surface display laccase biocatalysis system. (A) Schematic depicting the surface-display laccase biocatalysis approach for organic contaminant degradation. (B) Design of an engineered cell for laccase surface-display on the yeast host cell. The laccase enzyme is fused with a cell wall anchoring protein a-agglutinin (with two subunits Aga1p/Aga2p). The Aga2p subunit is secreted, while the Aga1p subunit is incorporated into the cell wall, and the two are linked via two disulfide bonds. Thus, laccase is immobilized (and thus "displayed") on the cell surface as a fusion with the Aga2p subunit. The displayed laccase was also fused to a red fluorescent protein DsRed-Monomer on its *C* terminus to allow for protein visualization. *GAL1p*: *GAL1* inducible promoter, *AGA1/AGA2*: the genes encoding the two subunits of a-agglutinin Aga1p/Aga2p, LAC3: the gene encoding laccase from *T. versicolor,* rfp: red fluorescent protein encoded by DsRed-Monomer gene.

and subsequently shown to couple the reduction of molecular oxygen with the oxidation of a broad range of organic substrates, including phenols, polyphenols, methoxy-substituted phenols, and aromatic and aliphatic amines.[17,18] Prior studies have demonstrated the activity of fungal laccases in degrading various emerging contaminants, such as bisphenol A (BPA),[19,20] estrogens, (e.g., estrone (E1)[21,22] 17β-estradiol (E2),[21] 17α-ethinylestradiol (EE2),[23] atrazine,[24] sulfamethoxazole,[25] and other persistent organic contaminants.[26] While biocatalysis by laccases holds great potential for organic compound transformation, use of laccase biocatalysis for water and wastewater treatment[23,27] is underexplored compared to its use for industrial applications such as textile-dye or pulp bleaching, food improvement, polymer synthesis, and the development of biosensors.[28]

A successful system using enzyme biocatalysis must ensure enzyme functionality and stability while maximizing catalytic efficiency. Simple reuse and regeneration of biocatalysts is also critical to implementing cost-effective, continuous treatment processes.[18] Biological enzymes have been tested for water treatment in the solution form,[23] encapsulated form,[29] or after immobilization in matrices.[30,31] Using enzymes in solution as additives for water treatment is not suitable and economical due to short enzyme lifetimes, challenging recovery, and high cost of single use.[32] Immobilization of enzymes in solid matrices such as porous glass beads, alginate-carbon composite beads, organic gels, and cross-linked aggregates have demonstrated enhanced enzyme resistance to inhibitory or denaturing conditions and ability of enzyme reuse.[33] However, these immobilization techniques have major drawbacks such as loss of enzyme functionality due to denaturation by chemical processing and limitations in substrate mass transfer.[30] In contrast to these earlier studies, the present study implemented a distinct strategy that immobilizes the laccase enzyme on the outer surface of biological cells to develop a new form of biocatalyst that overcomes the above drawbacks (Figure 1A).

With cell surface display technology, functional enzymes are expressed and displayed on the outer surface of microbial host cells by fusion with cell wall or plasma membrane using molecular biology and enzyme engineering techniques.[34] An intensively studied host cell system for enzyme surface display is the Baker's yeast *Saccharomyces cerevisiae*.[35] *S. cerevisiae* has been granted "generally regarded as safe" (GRAS) approval by the Food and Drug Administration and is suitable for industrial and food use. Various heterologous enzymes have been displayed on the yeast cell surface, which has potential applications in biosensors, biofuel production, drug screening, and metal adsorption.[36] Cell surface display assures low mass transfer resistance and high enzyme accessibility while also providing a stable matrix for enzyme immobilization as well as simple regeneration and recovery. Fungal laccases would be suitable for cell surface display, because it does not rely on an intracellular environment to maintain activity and only needs oxygen (without other biological cofactors) for biocatalysis to occur. Therefore, we reasoned that surface display of fungal laccases could be exploited as an effective and efficient biocatalysis treatment strategy that combines the advantages of the high activity of free enzymes with the process advantages of immobilization.

In the present study, a novel surface-display laccase (SDL) biocatalyst was constructed by expressing laccase from the white-rot fungi *Trametes versicolor* on the yeast cell surface (Figure 1). Enzyme activity and localization in the SDL biocatalyst were confirmed through enzymatic assay and fluorescence microscopy imaging. In addition, characterization results demonstrated that the SDL biocatalyst had high longevity and stability compared to free laccase, could be separated and recovered from reaction solutions, retained high activity in reuse, and could be regenerated simply by cell cultivation. Finally, proof-of-concept evaluation of the SDL in treating recalcitrant organic contaminants of concern was performed with bisphenol A (BPA, an EDC) and sulfamethoxazole (SMZ, an antibiotic). Both of these emerging contaminants are among those most frequently detected in natural aquatic environments.[4,37] BPA is a widely used plastic monomer and plasticizer for the production of polycarbonate and epoxy products.[38] The proven estrogenic activity of BPA can lead to testicular/breast cancer, reproductive impairments,

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and other medical disorders for human beings, as well as cause physiological abnormalities in wildlife.[39,40] SMZ is an important sulfonamide antibiotic applied in the treatment of gastrointestinal infections,[41] and it exhibits low affinity to most soils and greater mobility than other antibiotics.[42] BPA and SMZ were tested as model substrates for SDL biocatalysis in this study to determine the efficacy of the laccase enzyme after surface display. This study reports, for the first time, the development of surface display enzyme biocatalysts as an effective and renewable alternative for treating persistent organic pollutants in water. As fungal laccases have been found to degrade various emerging contaminants,[28] our results suggest a potential broadly applicable contaminant treatment strategy using this new type of SDL biocatalyst.

MATERIALS AND METHODS

Strains, Medium, and Chemicals. The *S. cerevisiae* strain EBY100 (MATa *ura* 3-52 *trp* 1 *leu*2Δ1 *his*3Δ200 *pep*4:*HIS*3 *prb*1Δ1.6R *can*1 *GAL*) was obtained from ATCC (Manassas, VA) and was used in this study as the carrier host cell for enzyme surface display. *Escherichia coli* TOP10 strain was used for gene cloning and manipulation. *E. coli* cells were regularly grown in Luria−Bertani medium at 37 °C, and 100 μg/mL of ampicillin was added to the medium when required. The wide type EBY100 strain was cultivated at 30 °C in YPD medium (10 g/L of yeast extract, 20 g/L of peptone, and 20 g/L D-glucose). BPA (bisphenol A, ≥ 99%), sulfamethoxazole, D-glucose, D-(+)-galactose, 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS, 99%), and purified *Trametes versicolor* laccase were purchased from Sigma-Aldrich (St. Louis, MO). Restriction enzymes, ligase, and molecular reagents for polymerase chain reaction (PCR) were obtained from New England Biolabs (Beverly, MA). Primers for sequencing were synthesized by Integrated DNA Technologies (Coralville, IA). The strains and primers in this study are listed in Table S1 (Supporting Information). All other general chemicals and medium components were supplied by Fisher Scientific (Pittsburgh, PA).

Construction of Plasmids for Cell Surface Display of Laccase. Information on plasmids in this study is summarized in Table S1 (Supporting Information). The laccase encoding gene *LAC3* from *T. versicolor* was codon optimized for the host cell *S. cerevisiae* for functional expression. The codon optimized *LAC3* was synthesized by Genscript (Piscataway, NJ). The first 63 nucleotides (encoding the signal peptide), as well as the stop codon of the *LAC3* gene, were removed, and a *Nhe*I restriction site was added to the 5′ end of the gene and a *Bam*HI site was added to the 3′ end. Next, the fusion protein cassette, which contains the *N*-terminal secretion signal sequence of the Aga2p subunit of the cell wall anchoring protein a-agglutinin in the yeast and modified *LAC3* gene sequence, was constructed by ligating to the pCTcon2 plasmid (Supporting Information, Figure S1A) (obtained from Addgene (Cambridge, MA)), yielding the plasmid pCTcon2-Lac3. To allow for protein characterization and visualization, the Discosoma sp. red fluorescent protein (DsRed) monomer sequence was inserted into the pCTcon2-Lac3 plasmid by blunt end cloning so that the displayed Lac3p was fused with DsRed-Monomer on its *C* terminus. The resulting plasmid was named pCTcon2-Lac3-DsRed (Supporting Information, Figure S1B). The correct construction of the plasmids was confirmed through Sanger sequencing.

Yeast Transformation. The plasmid pCTcon2-Lac3 was transformed to the *S. cerevisiae* strain EBY100 for laccase surface display. The control plasmid pCTcon2 (without *LAC3* gene) was transformed to EBY100 to obtain the control strain without laccase surface display. The plasmid pCTcon2-Lac3-DsRed was transformed for visualizing laccase expression. Plasmid extraction was performed using the QIAprep Spin Miniprep Kit (Germantown, MD). Yeast transformation was conducted using the LiAc/PEG method described previously.[43] Yeast transformants were then selected on synthetic complete medium without tryptophan (SC-trp medium) containing 6.7 g/L of yeast nitrogen base, appropriate amino acids without tryptophan, 20 g/L D-glucose, and 20 g/L agar.

Surface Display Laccase (SDL) Biocatalyst Preparation by Cell Cultivation. The SDL biocatalyst cells or the wild type control cells were cultured in SC-trp medium containing 20 g/L glucose to early stationary growth phase. Cells were harvested by centrifugation and transferred into new SC-trp medium containing 20 g/L galactose, 2 g/L glucose, and 0.1 mM $CuSO_4$ for protein expression induction by cultivating at 30 °C and 250 rpm for 18 h (OD_{600} ∼ 3−4). The induced cells were harvested by centrifugation and washed twice with 0.1 M acetate buffer (pH = 5). The induced cells were suspended in acetate buffer to appropriate density and used as a homogeneous biocatalyst source for experiments.

Fluorescence Microscope Imaging. To confirm the expression and localization of laccase-DsRed monomer fusion protein on the yeast cell surface, the SDL yeast cells were visualized on an Eclipse 90i fluorescence microscope (Nikon Instruments Inc., Melville, NY). The red fluorescence signal from DsRed monomer was detected through a U-MWIG2 mirror unit with a BP520-550 excitation filter, DM565 dichroic mirror with BA580IF emission filter (Olympus). Images were analyzed with MetaMorph software (MDS, Inc., Sunnyvale, CA).

Laccase Enzyme Activity Assays. The enzyme activity of free laccase and SDL biocatalyst were determined by measuring the oxidation rate of 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS, a model substrate for laccase) using a colorimetric method.[44] The reaction was set up by adding 25 μL of the sample to a mixture consisting of 35 μL of 5.0 mM ABTS and 190 μL of 0.1 M acetate buffer (pH = 5) in 96-well microtiter plate wells. The absorbance at 420 nm was measured every 2 min for 30 min in a Synergy HT Microplate Reader (BioTek, Winooski, VT) at 37 °C under continuous shaking. The catalytic oxidation rate of ABTS by laccase could be calculated by determining the ABTS absorbance change over time. One unit (U) of enzyme activity is defined as the amount of enzyme required to catalyze 1 μmol substrate per minute.[45] All measurements were conducted in triplicate.

Bisphenol A and Sulfamethoxazole Degradation Experiments. The BPA and SMZ degradation experiments were performed with SDL biocatalyst. Appropriate amounts of SDL corresponding to 0.25 U/mL were used for BPA (60 μM) and SMZ (30 μM) degradation experiments, in a 5 mL solution containing 0.1 M sodium acetate buffer (pH = 5) in each 14 mL test tube. The tubes were incubated at 37 °C and 250 rpm, and liquid samples were taken periodically for measuring contaminants concentrations. 0.05 U/mL and 0.02 U/mL SDL biocatalyst was added to degrade 2 μM and 2 nM BPA, respectively. Reactions without SDL amendment were set up as the abiotic control, and reactions amended with yeast cells containing the plasmid backbone without expressing laccase

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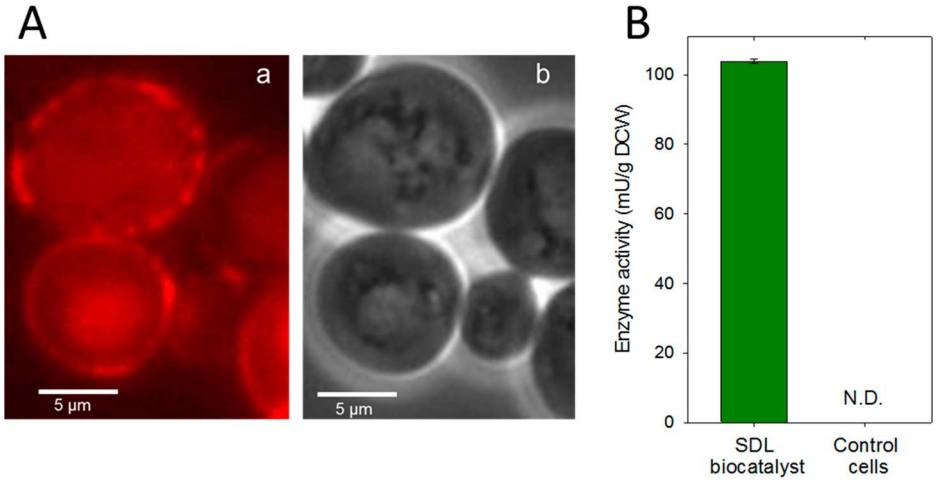

Figure 2. Laccase is functionally displayed on the yeast cell surface. (A) Microscopic observation of the engineered cells with surface-display laccase tagged with red-fluorescent protein. (a) Fluorescence micrograph shows localization of the laccase enzymes on the cell wall. (b) Phase-contrast micrograph of the same field of view. (B) Laccase enzyme activity of the SDL biocatalyst and control cells. One unit (U) is defined as the amount of enzyme required to catalyze 1 μmol substrate per minute. Results were normalized to dry cell weight (DCW).

recombinant protein (i.e., control cells) were used as the negative biological control. All experiments were set up in triplicate.

An actual wastewater sample was collected from the outlet of the secondary clarifier of the municipal wastewater treatment plant in South Bend, IN. The water sample was filtered (0.45 μm) and then stored at 4 °C until use. The water was analyzed according to standard methods,[46] and the characteristics are listed in Table S2 (Supporting Information).

Analytical Methods. Cell number (OD_{600}) was measured by using an UV−visible spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Concentration of BPA or SMZ was quantified by using high performance liquid chromatography (Agilent Technologies 1200 series) equipped with a UV detector under the wavelength of 274 nm and with an Eclipse XDB-C18 column (Agilent Technologies, Santa Clara, CA). The column was eluted with methanol and 2 mM acetic acid solution (v/v = 60:40) under the flow rate of 1 mL/min at 25 °C. The concentrations of BPA in μg/L levels were analyzed by using BPA ELISA kit (Detroit R&D, Detroit, MI) and following the manufacturer's instructions.

RESULTS AND DISCUSSION

Fungal Laccase Was Functionally Expressed on the Yeast Cell Surface. Functional expression and surface display of the *T. versicolor* laccase Lac3p in the host cell *S. cerevisiae* was achieved by constructing the plasmid pCTcon2-Lac3-DsRed (Supporting Information, Figure S1) and transformation into the yeast. The synthetic surface display laccase (SDL) biocatalyst system is illustrated in Figure 1B. Specifically, the cell wall-anchoring protein a-agglutinin was used for cell surface display of the target enzyme Lac3p in the yeast. a-Agglutinin consists of Aga1p and Aga2p subunits. The Aga2p subunit is secreted, while the Aga1p subunit is incorporated in the cell wall, and the two are linked via two disulfide bonds. The a-agglutinin protein possesses an *N*-terminal secretion signal sequence for transport to the cell surface and *C*-terminal glycosylphosphatidylinositol (GPI) anchor attachment signal sequence for transient anchoring in the plasma membrane and later to the cell wall.[47] In the a-agglutinin based system utilized, Lac3p was fused with the C-terminus part of Aga2p, and the fusion protein was displayed on the cell surface.

To allow for protein characterization and visualization, the displayed laccase was also fused to a red fluorescent protein DsRed-Monomer on its C-terminus. The constructed SDL biocatalyst showed a strong red-fluorescence signal on the cell surface (Figure 2A), indicating a successful surface display of laccase. We quantified enzyme activity of the SDL biocatalyst by measuring the oxidation rate of ABTS (a model substrate for laccase) using a colorimetric method.[44] ABTS is a model substrate for laccase, and it has been widely used as a standard compound to quantify laccase enzyme activity in the literature. The SDL biocatalyst had an enzyme activity of 104 ± 3 mU/g dry cell biomass, while no activity was detected for the control (cells without laccase expression) (Figure 2B). The results demonstrated that laccase was functionally expressed in the yeast host cell and retained its function when immobilized on the cell wall. We note that the red fluorescence signal was also detected inside the cells, although at a lower level than the cell surface. Possible reasons could be that the fusion protein was overexpressed and exceeded the capacity that the cell wall could immobilize or the GPI anchor on the fusion protein anchored into some intracellular membrane structures as well.[48] Meanwhile, it should be noted that the reaction supernatant (i.e., liquid without the SDL biocatalyst) had little enzymatic activity, suggesting that there was no laccase leakage from the SDL during the reaction (Supporting Information, Figure S2).

Surface Display Significantly Improved the Stability of Laccase. To access the enzyme stability of the surface display laccase, we determined enzyme activities of the SDL as well as the free form of laccase over time when the enzymes were stored at room temperature. The ABTS enzyme assay was conducted, and relative enzyme activity based on standardized enzyme unit was used for valid comparison. The activity of the SDL biocatalyst declined slowly in the first 10 days by about 10% and then appeared to stabilize, retaining more than 90% of the initial activity after 25 days (Figure 3). In contrast, the activity of free laccase declined to 60% of its initial activity after 25 days. It is worth noting that the SDL biocatalyst cell density did not change significantly during storage as there were no nutrients for cell growth. The yeast can remain viable with

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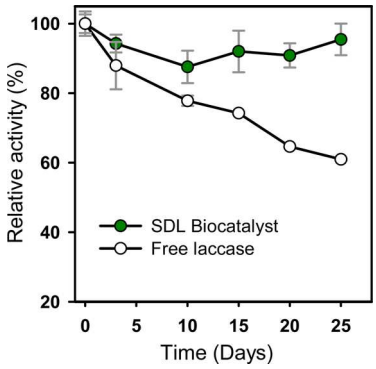

Figure 3. The surface displayed laccase (SDL) is more stable than free laccase in solution. Stability of SDL biocatalyst and free laccase was assayed over time (in acetate buffer pH 5, at room temperature), using aliquots. Results are the means of triplicate experiments; error bars indicating standard deviations are not visible when smaller than the symbol size.

limited growth under minimal nutrient conditions, and this could be an advantage for use as a robust host cell system under environmental conditions. Enhancement of enzyme stability or longevity by immobilization on other matrix materials has also been previously observed.[18,30,31] For example, a fungal laccase immobilized on electrospun fibrous membranes retained 70% of original activity, while the free enzyme had only 30% activity remaining after 30 days.[49] Based on our stability data, it is estimated that the half-life of the SDL was on the order of months (e.g., $t_{1/2}$ = 58 days estimated from data points within 10 days and assuming first-order decay) even with minimal optimization, therefore supporting the potential application of the biocatalyst in contaminant treatment.

The SDL Biocatalyst Could Be Reused Multiple Times. A primary motivation for enzyme immobilization is to enable reuse of the enzyme thus reducing operation cost in treatment processes. The SDL biocatalyst constructed on the yeast host cell could be viewed as particles with the size of 5−10 μm, coated with functional laccase on the surface, and it could easily be separated from the reaction solution by centrifugation, washed with the buffer solution, and then reused. To test the operational stability of the SDL during reuse, we examined the laccase activity when the SDL biocatalyst was used over multiple cycles of reactions. Figure 4A illustrates that the SDL oxidized eight batches of 0.7 mM model substrate ABTS while retaining 74% of initial activity at the end of the eighth reuse. Free laccase could not be readily reused because soluble enzyme could not be separated from solutions without complicated protein purification procedures such as protein ultrafiltration or column chromatography which usually has low recovery.[50]

With its reusability and operational stability, the surface display enzyme biocatalyst could be exploited as a catalytic biomaterial for developing cost-effective and robust enzyme biocatalysis processes. Different from conventional application of microbial cells, the SDL biocatalyst does not depend on cell growth to be functional; what matters is the enzyme catalytic activity and that the cell serve as a form of carrier for enzyme immobilization. To evaluate the potential of regenerating the used SDL biocatalyst, we conducted cell growth experiments with the SDL cells after eight repeated batch reactions in degrading ABTS. The used SDL cells grew at a rate similar to that of fresh cells (i.e., newly prepared cells without any reaction with ABTS) after inoculation into the culture medium (Figure 4B). The regenerated SDL had the same level of laccase enzyme activity as the fresh SDL shown in Figure 2B. The results suggest that the SDL biocatalyst could be conveniently and efficiently regenerated and amplified for extended reuse.

Surface Displayed Laccase Effectively Catalyzed BPA Degradation. To assess the potential of the SDL for organic contaminant degradation, the endocrine disrupting compound BPA was examined as a model substrate. Due to its wide use and only poor removal in WWTPs, BPA is commonly detected in wastewater effluents and natural environments.[38] It has been reported that the main transformation products of BPA by laccase catalysis were 4-isopropylphenol and phenol and the estrogenic activity could be effectively reduced with the oxidation of BPA in the presence of sufficient laccase enzyme.[51−53] Batch experiments were conducted in acetic acid buffer (pH = 5) containing 60 μM BPA, with or without the redox mediator ABTS. It was observed that BPA concentration decreased substantially by 43%−48% within 2

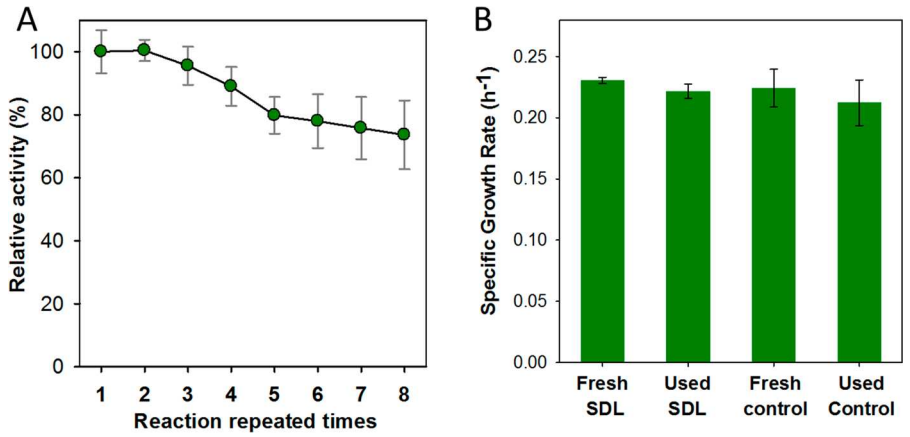

Figure 4. The SDL biocatalyst is reusable and regenerable. (A) Reusability, indicating the ability to recover and reuse the same batch of SDL biocatalyst, was evaluated in repeated batch reactions of ABTS oxidation and was found to be high. (B) Regenerability was assessed by measuring specific growth rate of cells recovered after eight times reuse in synthetic complete yeast growth medium containing 20 g/L glucose and comparing it to fresh cells grown in the same growth medium but not subjected to biocatalysis experiments. No significant change in specific growth rates was observed. Results are the means of triplicate experiments; error bars indicating standard deviations are not visible when smaller than the symbol size.

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min after adding the SDL biocatalyst cells (Supporting Information, Figure S3). Also, a similar level of BPA concentration reduction occurred in the control reaction amended with the wild type cells without laccase surface display at the 2 min time point, while there was no significant change of the BPA concentration in the abiotic control without cells. The observation indicated adsorption of BPA to the yeast cells. Despite the initial concentration drop, the BPA concentration in the control reaction amended with wild type cells did not change significantly after the first sampling point (i.e., after 2 min). In comparison, the removal of BPA continued in the reactions amended with the SDL biocatalyst (Supporting Information, Figure S3), indicating active enzymatic degradation. These results demonstrated that the SDL was functional and catalyzed the enzymatic degradation of BPA.

Since no further BPA removal occurred in the control reaction with wild type cells after the first sampling point (2 min), it is reasonable to assume that BPA adsorption saturated rapidly and that BPA concentration decrease after the first sampling point was largely due to enzymatic degradation. Thus, we used the following equation to determine BPA degradation efficiency due to laccase activity: degradation efficiency (%) = $\frac{C_1 - C_t}{C_1} \times 100\%$, where C_t is the BPA concentration at time t, and C_1 is the BPA concentration at the first sampling point which already takes into account the BPA decrease caused by adsorption. BPA degradation efficiency with SDL under the conditions with or without the redox mediator ABTS was determined. ABTS is a naturally occurring compound that can act as a redox mediator to facilitate laccase biocatalysis of recalcitrant organic compounds.[54] Particularly, if the structures of organic compounds of interest limit their access to the active site of laccases, they may not be readily oxidized by laccases. Nature overcomes the limitation of substrate access through laccase-mediator systems (LMS), where certain chemicals act as "electron shuttles" between the laccase and the substrate compounds.[28,55] Namely, redox mediators can be oxidized by laccases and be reduced back to their original species during oxidation of target organic compounds (Figure 5A). Noticeably, removal of BPA by the SDL was stimulated with the addition of ABTS (Figure 5B). Addition of 10 μM, 20 μM, and 30 μM ABTS increased the removal efficiency at 42 min to 42 ± 3%, 60 ± 3%, and 73 ± 4%, respectively, while the BPA removal efficiency with SDL alone was 22 ± 3%. The results suggested that direct interaction between BPA and active sites on the SDL might be a main rate limiting step for BPA degradation, which could be overcome by the addition of a redox mediator.

Additional experiments were conducted with 2 μM and 2 nM of BPA in buffer solutions to further evaluate the performance of the SDL under environmentally relevant concentrations. Under both conditions, adsorption of BPA observed after adding cells was less than 20%. The degradation efficiency was calculated using the equation described above. The degradation efficiency of BPA at 2 μM initial concentration with 0.05 U/mL SDL reached 23 ± 4% after 120 min, and addition of 2 μM ABTS significantly increased the degradation efficiency to 80 ± 5% (Supporting Information, Figure S4A). At 2 nM initial concentration with 0.02 U/mL SDL, 58 ± 6% BPA was degraded after 6 h, and addition of 2 nM ABTS enhanced the degradation efficiency to 93 ± 2% (Supporting Information, Figure S4B). The results demonstrated that the SDL biocatalyst was effective in degrading BPA at trace levels.

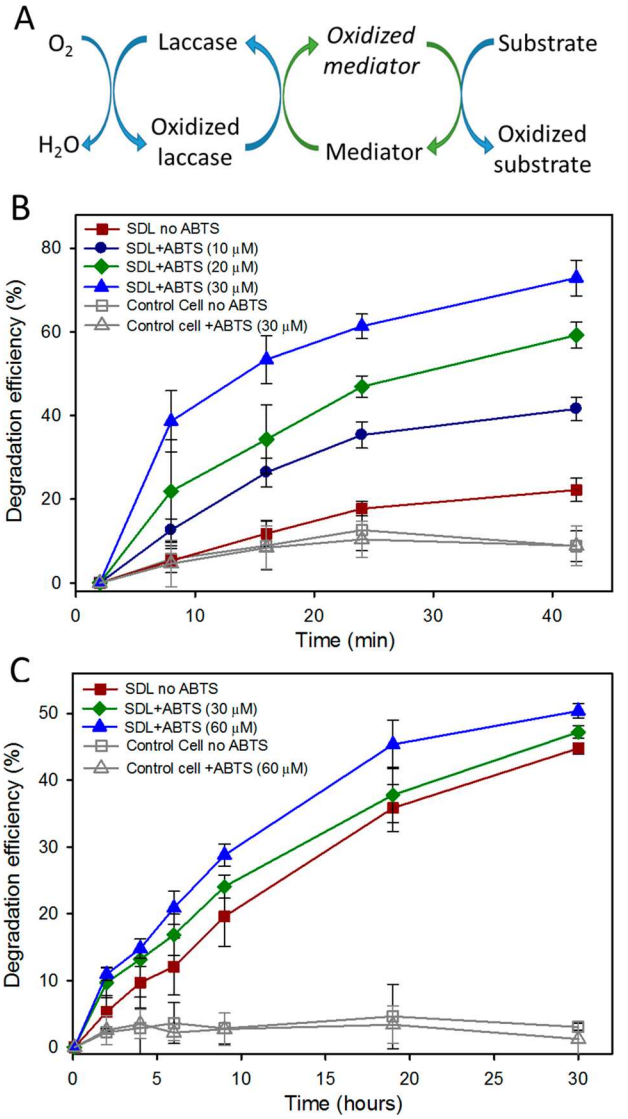

Figure 5. The SDL biocatalyst effectively degrades the organic pollutants bisphenol A (BPA) and sulfamethoxazole (SMZ). (A) Schematic of the laccase mediator system where laccase mediators promote enzyme biocatalysis through electron shuttling. (B) Degradation efficiencies of BPA by the SDL or control cells under conditions without redox mediator ABTS or with ABTS (10 μM, 20 μM, or 30 μM). (C) Degradation efficiencies of sulfamethoxazole (SMZ) by the SDL alone and the SDL or control cells under conditions without ABTS or with ABTS (30 μM or 60 μM). Results are the means of triplicate experiments; error bars indicate standard deviations. Reactions with control cells or control cells with the highest concentration of ABTS for either contaminant degradation experiment were set up as negative control.

We further performed batch experiments with filtered secondary wastewater effluent. BPA was amended to obtain an initial concentration of 2 nM. The degradation efficiency of BPA by the SDL biocatalyst reached 46 ± 6% with no ABTS and 82 ± 3% with ABTS after 6 h of reaction (Supporting Information, Figure S5), while the incubation with control cells had no significant BPA degradation.

Surface Displayed Laccase Effectively Catalyzed SMZ Degradation. To further assess the potential of the SDL for

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organic contaminant degradation beyond BPA, the antibiotic SMZ was examined as a model substrate. SMZ cannot be effectively removed by conventional biological wastewater treatment processes[56] and is among the most frequently detected pharmaceutical and personal care products in natural aquatic environments.[1,42] The intermediate degradation products of SMZ by laccase were reported to be dependent on the experimental conditions.[25,57] Despite the formation of various laccase-transformed products, in general, lower antibacterial activity was observed after treatment.[25,57−59] SMZ is much more hydrophilic than BPA; the Log K_{ow} for SMZ is 0.89, while the Log K_{ow} for BPA is 3.32.[60] Therefore, using SMZ as a substrate provides a distinct scenario to evaluate the biocatalytic ability of the SDL with little adsorption of the target contaminant. Recent studies have reported the ability of fungal laccases to degrade SMZ.[25,58] Batch experiments were conducted in acetic acid buffer (pH = 5) containing 30 μM SMZ. Our initial tests showed that adsorption of SMZ to yeast cells was insignificant. Thus, we used 30 μM as the SMZ concentration in the experiments so that the SMZ concentration level after saturated adsorption would be comparable to that for BPA (i.e., ∼30−35 μM BPA in solution after saturated adsorption of ∼60 μM BPA). Results of total removal efficiency of SMZ were shown in Figure S6 (Supporting Information). As expected, only a small fraction of SMZ (4%−8%) was removed in the control reaction with wild type cells, suggesting that SMZ reduction by adsorption was limited. SMZ was removed gradually over time in the reactions amended with the SDL biocatalyst. The total removal efficiency of 30 μM of SMZ by the SDL at 30 h reached 47 ± 1% without ABTS, which could be attributed to the SDL enzymatic activity.

The enzymatic degradation efficiency for SMZ was then calculated based on the equation described earlier for BPA degradation. As shown in Figure 5C, addition of the mediator, ABTS, increased the removal efficiency slightly with final removals after 30 h to 47 ± 1% and 50 ± 1% with 30 μM or 60 μM ABTS, respectively, while the SDL catalyst with no ABTS removed 44 ± 1% SMZ. It should be noted that adding 10 μM or 20 μM ABTS (the concentration used for BPA) did not significantly increase SMZ degradation efficiency (data not shown), and thus we increased the concentration range of ABTS up to 60 μM. Similar results were observed for degradation of 30 μM SMZ amended in filtered secondary wastewater effluent (Supporting Information, Figure S7). The SDL biocatalyst degraded 38 ± 2% SMZ without ABTS and 50 ± 3% with the addition of 60 μM ABTS. The marginal increase in SMZ degradation efficiency by ABTS addition were in contrast to the observation in BPA degradation, indicating that access of the SMZ molecule to the active laccase enzyme sites on cell surface was not as severe a limiting factor as that for the BPA degradation. Results demonstrated that addition of laccase mediators could be an effective strategy to enhance efficiency of the SDL, but the extents of improvement may vary for different contaminants. It is worth noting that a variety of organic compounds were found to be laccase redox mediators. Typically, a redox mediator can be oxidized by laccase, and the oxidized form has a redox potential higher than the target compound.[61] While ABTS is the most widely used model mediator, many other natural phenolic compounds (e.g., humid acids) have also been identified to be effective laccase mediators[62,63] and could be used to enhance laccase catalytic capacity.

Kinetics Analysis and Discussion on BPA and SMZ Degradation by the SDL. To further examine BPA and SMZ degradation by the SDL, the kinetic order was determined by regression analysis, and the kinetic rate constants (k) were extracted (Supporting Information, Table S3). Comparing the degradation rate of BPA and SMZ, we observed that BPA degradation was much faster than SMZ, because SMZ was intrinsically more persistent to degradation by laccase than BPA.[64] Noticeably, both BPA and SMZ degradation by the SDL fit to a second-order reaction model. In general, an enzyme reaction follows first-order reaction kinetics in the low substrate concentration range or zero-order reaction kinetics in the high substrate concentration range.[65] Consistent with this general rule, BPA degradation experiments with free laccase followed the first-order reaction (Supporting Information, Figure S8). However, change of reaction order is not uncommon for immobilized enzymes. For example, a previous study reported that fatty acid production from glycerolysis by immobilized lipases was a second-order reaction.[66] Another study observed a third-order reaction for PAH removal by fungal laccase immobilized on electrospun fibrous membranes.[49] The change of the kinetic order for immobilized enzymes might be attributed to conformational and steric effects, partitioning effects, microenvironmental effects, and mass-transfer effects.[67−69] Immobilization in general may greatly alter the properties of the enzyme surroundings, creating more hydrophobic or hydrophilic environments around the enzyme and thus affecting substrate accessibility.[70] The mass-transfer, i.e., adsorption and/or diffusion of substrate to the active site of the enzyme often occurs as the reaction-rate limiting step and thus directly causes the change in kinetic order.[49]

The different effects of ABTS addition on stimulating degradation rates of BPA and SMZ were probably related to the distinct adsorption properties of the two compounds. BPA is more hydrophobic than SMZ and thus was more easily adsorbed to SDL cells (e.g., hydrophobic components of the yeast cell wall). Initial adsorption of BPA would accumulate high concentration of BPA in the local microenvironment near the cell surface, resulting in decreased diffusion of BPA from bulk solution to the cell surface and thus less chance of BPA to access the active site of the surface displayed laccase. ABTS addition could bypass the need for contact between BPA and laccase and facilitated BPA degradation through shuttling electrons between BPA and the SDL. In comparison, since there was little adsorption of SMZ to cells, access of SMZ to surface-displayed laccase was not limiting, and thus addition of ABTS did not show considerable stimulating effects. Further studies could investigate the kinetics and substrate mass transfer for the SDL and redox mediator system in greater detail.

It should be noted that the SDL biocatalyst reported in this study could be optimized further to be a more effective remediation biocatalyst. Future work will focus on comprehensively characterizing biocatalytic properties of the SDL, such as enzyme reaction kinetic parameters (the Michaelis−Menten constant which is a measure of substrate affinity, K_m; the turnover number, k_{cat}; and the catalytic efficiency of the enzyme (k_{cat}/K_m)). Such information would help identify what kinetic parameters are most likely to limit the activity of the biocatalyst, providing a focus for optimization.

Environmental Implications. In this study, we have constructed a surface-display fungal laccase biocatalyst using

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synthetic biology tools and demonstrated its effectiveness for treating persistent organic micropollutants in water for the first time. The SDL biocatalyst had high longevity and stability, could be recycled and reused in multiple treatment processes, and could be produced and regenerated by simple cell cultivation. We envision the use of SDL as a polishing step in wastewater reuse scenarios. This innovative treatment technology can be either operated alone (e.g., advanced treatment of tertiary effluent to degrade persistent organic pollutants, treatment of industrial wastewater) or in combination with current advanced water treatment technologies. The SDL biocatalysis could also potentially be used to remove organic contaminants in reverse osmosis concentrate, a challenging waste stream in inland desalination and water reuse.[71,72] A membrane bioreactor, where the SDL biocatalyst is contained, could be a possible treatment configuration for developing this new technology. In addition, by changing the target enzymes for expression in the surface display platform system, new biocatalysts with various desired functions could be created and applied for other components of the food-water-energy nexus. For example, one potential application would be nutrient removal and recovery by surface displaying P binding enzymes[73,74] and/or N transforming enzymes.[75,76]

Our study showed that adding ABTS could enhance contaminant degradation efficiency by the engineered laccase biocatalyst. However, the potential toxicity of redox mediators needs to be carefully considered in real applications.[25,77] The toxicity issue of synthetic redox mediators could be overcome by screening natural redox mediators which are harmless, ecofriendly, and of low cost.[78] Through screening of a variety of compounds generated during natural degradation of lignin by white-rot fungi, several efficient natural redox mediators with low toxicity have been identified and could be exploited for treatment applications.[79,80]

Engineered biocatalysts hold great promise for beneficial biotransformation,[35,81] but the assumption of potential ecological risks impedes exploration of genetically engineered biocatalysts in environmental engineering applications. Concerns primarily arise from the possibility of horizontal gene transfer and corelease of antibiotic resistance marker genes.[82] However, these concerns can now be minimized by using the advanced genome editing technique CRISPR-Cas9,[83] which can achieve stable and seamless genome integration of genes of desired enzymes without employing any unwanted antibiotic resistance marker genes. The functional laccase expressing genetic cassette demonstrated in this study can be readily integrated into the yeast genome by implementing the CRISPR-Cas9 technique (which has been well developed for *S. cerevisiae*[84,85]) to construct a stable and marker-free SDL. This means that the engineered biocatalyst will not have risks of causing antibiotic resistance or horizontal gene transfer, thus minimizing possible environmental risks.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b01641.

Strains, plasmids, and primers used in this study (Table S1), characteristics of the secondary effluent (Table S2), kinetics analysis of BPA and SMZ degradation (Table S3), schematic of plasmids (Figure S1), enzyme activity of the SDL and cell-free supernatant (Figure S2), overall BPA removal (Figure S3), trace level BPA degradation (Figure S4), trace level BPA degradation amended in filtered secondary wastewater effluent (Figure S5), overall SMZ removal (Figure S6), SMZ degradation amended in filtered secondary wastewater effluent (Figure S7), and BPA degradation kinetics by free laccase (Figure S8) (PDF)

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Notes

The authors declare no competing financial interest.

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