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Tyrosinase-functionalized polyhydroxyalkanoate bio-beads as a novel biocatalyst for degradation of bisphenol analogues

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ABSTRACT

Bisphenol compounds are emerging contaminants of high concerns with known endocrine-disrupting effects. Biocatalysis provides a green chemistry alternative for advanced treatment in water reclamation. This study created a novel biocatalyst through genetically immobilizing the *Bacillus megaterium* tyrosinase enzyme (BmTyr) on the surface of self-assembled polyhydroxyalkanoate (PHA) biopolymer beads (termed PHA-BmTyr) by using synthetic biology techniques and demonstrated one-pot in vivo production of the biocatalyst for effective degradation and detoxification of various bisphenol analogues for the first time. The degradation pathway of bisphenols was determined to be mediated by the monophenolase and diphenolase activity of BmTyr. Notably, biocatalytic bisphenol degradation by PHA-BmTyr could substantially reduce or eliminate estrogenic activity of the contaminants, and the degradation products had remarkably lower acute and chronic toxicity than their parent compounds. Furthermore, the PHA-BmTyr biocatalyst had high reusability for multiple bisphenol degradation reaction cycles and showed excellent stability that retained 100% and 86.6% of the initial activity when stored at 4 °C and room temperature, respectively for 30 days. Also, the PHA-BmTyr biocatalyst could efficiently degrade bisphenol analogues in real wastewater effluent matrix. This study provides a promising approach to develop innovative biocatalysis technologies for sustainable water reclamation.

1. Introduction

Bisphenols, a group of chemicals containing two hydroxyphenyl groups in their structure, have raised great concerns owing to their widespread environmental occurrence and potential ecotoxicological impacts (Chen et al., 2016; Liu et al., 2021). Among the bisphenol analogues, bisphenol A (BPA) has the highest annual production exceeding 4.7 million tons and is widely used in manufacturing various industrial and consumer goods, leading to its ubiquitous presence in diverse environmental matrices, especially aquatic systems (Belfroid et al., 2002; Jin and Zhu, 2016). BPA is a notorious endocrine disruptor that can result in reproductive dysfunction and developmental abnormality in humans and other organisms (Siracusa et al., 2018). Due to its health risks, BPA is increasingly replaced by other bisphenol analogues with structural similarity, including bisphenol B (BPB), bisphenol E (BPE), bisphenol F (BPF), and bisphenol S (BPS) (Liu et al., 2021). These bisphenol analogues have also been detected widely in different water environments (Noszczyńska and Piotrowska-Seget, 2018; Zhang et al., 2019). Moreover, many of these substitutive bisphenol analogues were found to possess comparable or even stronger endocrine disrupting effects than BPA (Cao et al., 2017; Liang et al., 2020; Rosenmai et al., 2014; Serra et al., 2019; Siracusa et al., 2018), whereas the techniques for their removal are still inadequately investigated.

There is an urgent need to develop innovative approaches for effective and efficient removal of bisphenols in wastewater treatment and reclamation, as wastewater treatment plants (WWTPs) are found to be a main source for discharge of bisphenols into aquatic environments (Wang et al., 2019a). The conventional treatment processes are inadequate to efficiently remove bisphenol analogues. For example, BPA, BPB, and BPE are recalcitrant in WWTPs with the average removal efficiencies of 41.6%, 34.1%, and 36.3%, respectively, and their concentrations could be up to micrograms per liter in the effluents (Wang et al., 2019a; Xue and Kannan, 2019). To tackle this challenge, advanced oxidation processes (AOPs) have received great research interest recently for removal of bisphenols (Hunge et al., 2021; Seibert et al., 2020). However, AOPs have major drawbacks such as requirement of high energy input, consumption of costly chemical reagents, and generation of undesired and more harmful byproducts (Miklos et al., 2018;

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Plahuta et al., 2014). Therefore, it is critically important to develop novel advanced treatment technologies for economical and environmentally sustainable remediation of bisphenol contamination in water reclamation.

Biocatalytic remediation, which capitalizes on enzymatic degradation and transformation of contaminants, is an emerging and promising technology for environmental pollution control (Eibes et al., 2015; Routoula and Patwardhan, 2020). Enzymes have high activity and specificity under ambient conditions, enabling low energy demands and chemical use for biocatalytic processes (Sheldon and Woodley, 2018). A broad repertoire of enzymes, particularly oxidoreductases and hydrolases, have been investigated for contaminant degradation applications (Strong and Claus, 2011; Zhu and Wei, 2019). Tyrosinase (EC 1.14.18.1), a type III copper enzyme found in many species and involved in betalains and melanin biosynthesis (Sanchez-Ferrer et al., 1995), can catalytically oxidize phenol and its derivatives coupled to reduction of molecular oxygen to water (Ba et al., 2014; Ikehata and Nicell, 2000). The biotechnological applications of tyrosinases have been reported in food and pharmaceutical fields, but the potential of this enzyme in environmental applications remains largely underexplored. Given the activity of tyrosinase towards phenolic compounds, we envision the enzyme could be a promising candidate for biocatalytic removal of bisphenols, but relevant research is limited.

Meanwhile, to achieve efficient and cost-effective biocatalytic treatment processes in water reclamation, it is critical to develop innovative biocatalysts with desirable functionality, high stability, and reusability. Because free enzymes are non-reusable and easily get inactivated, immobilization of enzymes is commonly used to achieve the aforementioned characteristics for practical applications (Zhu et al., 2019). However, conventional immobilization methods usually require laborious and costly protein purification, complicated chemical processing to immobilize the target enzyme to support materials, which could cause loss of protein functionality (Zdarta et al., 2018). To overcome these challenges, a novel platform technique, allowing for concurrent protein production and immobilization on the polyhydroxyalkanoate (PHA) bioplastic particles by using synthetic biology, has been introduced recently (Wong et al., 2020). PHAs are solid intracellular biopolymer naturally produced by some bacteria for carbon storage under unbalanced nutrient conditions (Moradali and Rehm, 2020). With a typical size of 50–500 nm, PHA particles have a core-shell structure where the hydrophobic core is composed of poly(3-hydroxybutyric acid) and the surface is coated with PhaC protein as the shell (Moradali and Rehm, 2020). By rational genetic engineering of PhaC protein, functional PHA bio-beads with the target enzyme immobilized on the surface can be constructed. Notably, this approach enables enzyme production, PHA particle formation, and immobilization process to occur concurrently within the host bacterial cells in a one-pot manner, eliminating the above-mentioned limitations of conventional methods. The PHA display platform has been exploited to immobilize enzymes such as lipase and amylase for industrial biotechnology applications (Blatchford et al., 2012; Jahns and Rehm, 2015; Rasiah and Rehm, 2009), and also has proven its feasibility and advantages in biomedical applications. However, its application in environmental pollutant degradation remains largely untapped. We envisioned this technology could also be exploited to develop effective and robust enzyme biocatalysts for contaminant removal in environmental remediation applications.

In this study, we developed a novel biocatalyst based on PHA display of tyrosinase enzyme for contaminant degradation applications in water reclamation. Specifically, we engineered *Escherichia coli* cells to express and display the *Bacillus megaterium* tyrosinase (BmTyr) on the surface of PHA bio-beads, referred to as PHA-BmTyr, through a simple one-pot

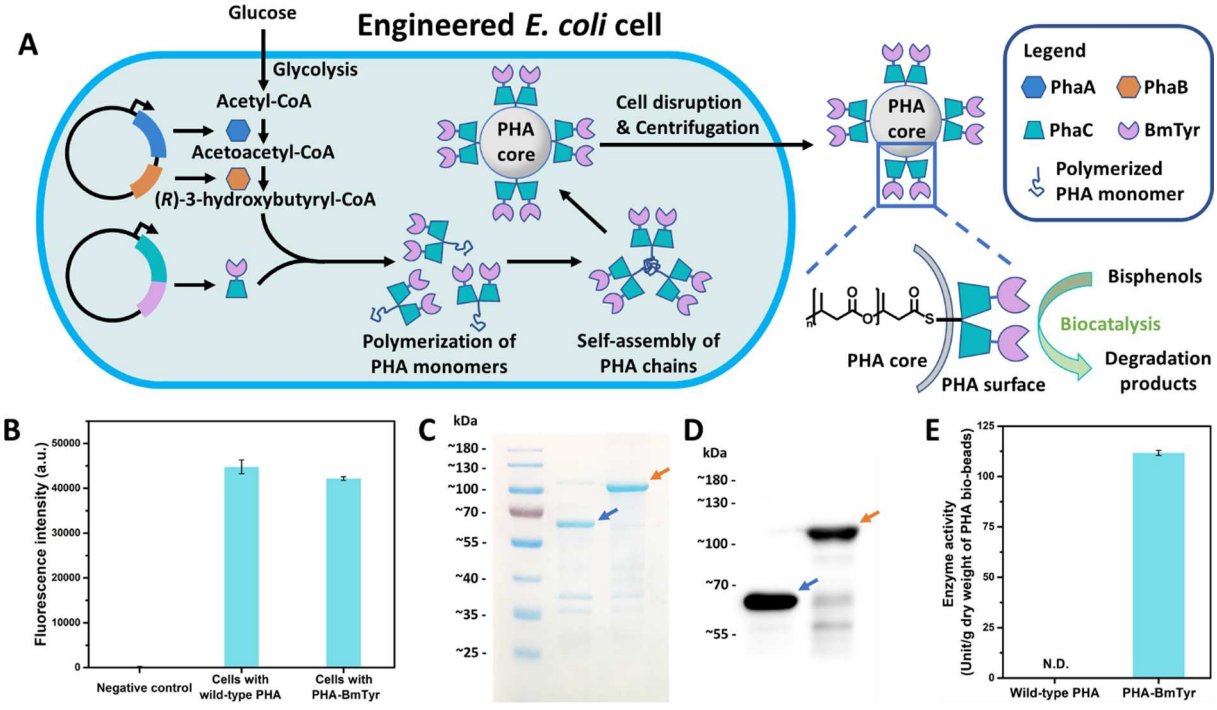

Fig. 1. Production and characterization of the PHA-BmTyr biocatalyst. (A) Schematic overview of design and production of the PHA-BmTyr biocatalyst for bisphenol degradation. Three enzymes were engaged including PhaA which is a β-kethothiolase, PhaB which is an acetoacetyl-CoA reductase and PhaC which is a PHA synthase. (B) Fluorescence intensity measurement of the Nile red staining assay. Negative control represents the *E. coli* cells without PHA bio-beads. The experiments were conducted in triplicates and values represent the mean ± standard error. (C) SDS-PAGE for the isolated PHA bio-beads. The blue and orange arrows indicate the bands of wild-type PhaC and PhaC-BmTyr fusion, respectively. (D) Western blot for isolated PHA bio-beads. The blue and orange arrows indicate the bands of wild-type PhaC and PhaC-BmTyr fusion, respectively. (E) Enzyme activity of PHA-BmTyr normalized to the dry weight of PHA bio-beads. N.D. denotes not detectable. The experiments were conducted in triplicates and values represent the mean ± standard error. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

production process (Fig. 1**A**). The PHA-BmTyr biocatalyst could effectively degrade various bisphenol analogues. Molecular docking was used to investigate the binding ability of the substrate to the BmTyr active site to explain the variations in degradability of different bisphenols. We characterized degradation intermediates and products and proposed the catalytic degradation pathway of bisphenols. Notably, the enzymatic degradation remarkably detoxified the hazardous bisphenols. Furthermore, the PHA-BmTyr biocatalyst had high storage stability and reusability, and it could efficiently degrade bisphenols in real wastewater effluent, suggesting its feasibility for practical remediation applications. Results from this study will contribute to advancing the development of innovative biocatalysis technology for sustainable water treatment and reclamation.

2. Materials and methods

2.1. Chemicals and reagents

BPA, BPF and BPS were purchased from Sigma-Aldrich (St. Louis, MO). BPB and BPE were purchased from TCI (Tokyo, Japan). All of the bisphenol compounds have the purity >98%, and their chemical structures and physicochemical characteristics are listed in **Table S1**. Nile red and isopropyl β- D-1-thiogalactopyranoside (IPTG) were obtained from Fisher Scientific (Pittsburgh, PA). 17β-Estradiol and 3,4-dihydroxy-L-phenylalanine (L-DOPA) were supplied by Sigma-Aldrich (St. Louis, MO). Restriction endonuclease, T4 DNA Ligase, Q5® High-Fidelity DNA Polymerase, and other reagents used in cloning work were purchased from New England Biolabs (Beverly, MA).

2.2. Bacterial strains and cultivation conditions

The bacterial strains used in this study are summarized in **Table S2**. *E. coli* TOP10 was used in general cloning work for plasmid construction, and *E. coli* BL21 (DE3) was used as workhorse cells for production of wild-type PHA bio-beads and BmTyr-functionalized PHA bio-beads. For cloning and plasmid propagation purposes, *E. coli* TOP10 cells were cultivated in Luria−Bertani (LB) medium supplemented with ampicillin (100 μg/mL) or kanamycin (50 μg/mL) as appropriate at 37 °C with 260 rpm shaking for 16 h.

2.3. Plasmid construction and production of PHA bio-beads

The details of plasmids used in this study are shown in **Table S2**. The gene encoding the tyrosinase from *B. megaterium* was synthesized by Genscript (Piscataway, NJ) with codon-optimization for *E. coli* expression. The genes *phaA*, *phaB*, and *phaC* from *Ralstonia eutropha* H16 were amplified from pBHR68 using high-fidelity PCR. The genes *phaA* and *phaB* were cloned into pBBR1MCS-2 under the control of a *lac* promoter to obtain the recombinant plasmid pBBR1MCS-2-PhaAB. The pET14b-PhaC-BmTyr was constructed by a 3-way ligation of the *phaC* gene with 5′ *Nde*I and 3′ *Spe*I sites, the gene encoding BmTyr with 5′ *Spe*I and 3′ *Bam*HI sites, and the pET14b backbone linearized by the *Nde*I and *Bam*HI restriction enzymes. The *phaC* gene was inserted into the pET14b backbone to construct the control plasmid pET14b-PhaC. The correctness of the recombinant plasmid construction was confirmed by Sanger sequencing.

The *E. coli* BL21 (DE3) cells were co-transformed with pBBR1MCS-2-PhaAB and pET14b-PhaC-BmTyr or with pBBR1MCS-2-PhaAB and pET14b-PhaC for production of BmTyr-functionalized PHA or wild-type PHA bio-beads, respectively. The transformant cells were cultivated in LB medium supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin at 30 °C and 260 rpm overnight. Then, the cells were inoculated into fresh LB medium containing 1% (w/v) glucose, 100 μg/mL ampicillin, and 50 μg/mL kanamycin and grown at 37 °C and 260 rpm. When the OD_{600} reached 0.5–0.6, the production of PHA bio-beads was induced with 1 mM IPTG at 25 °C and 230 rpm for 48 h. Afterwards, the cells were harvested and resuspended in 50 mM phosphate buffer (pH = 7) for disruption using ultrasonication. The PHA bio-beads were isolated and recovered by centrifugation at 16,000 *g* for 15 min and then washed twice using 50 mM phosphate buffer as described previously (Evert et al., 2020; Wong and Rehm, 2018). The recovered BmTyr-functionalized PHA bio-beads were subsequently exposed to 1.5 mM $CuSO_4$ in 50 mM Tris-HCl buffer (pH = 7) and mixed for 15 min at room temperature, followed by being washed twice to remove unbound copper ions and stored in 50 mM phosphate buffer. In this way, the BmTyr could bind and accommodate the copper ions at its active site to form a holoenzyme with full function (Shuster and Fishman, 2009).

2.4. Characterization of PHA bio-beads

A variety of methods were used to characterize the PHA bio-beads, including Nile red staining, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, scanning electron microscopy (SEM), and dynamic light scattering (DLS). The detailed characterization methods are provided in the SI.

2.5. Enzyme activity assay

The enzyme activity of PHA-BmTyr was measured using L-DOPA, a typical substrate for tyrosinase activity test, according to a published method (Kanteev et al., 2013). Briefly, 20 μL of PHA-BmTyr suspension was added into 180 μL of 50 mM phosphate buffer (pH = 7) containing 2 mM L-DOPA. The formation of L-dopachrome ($\varepsilon = 3600\ M^{-1}\ cm^{-1}$) from the oxidation of L-DOPA by BmTyr was monitored at 30 °C by measuring the absorbance at 475 nm at 2-min intervals for 30 min in a microplate reader (Synergy HT, BioTek, Winooski, VT). The enzyme activity was calculated and presented as unit (U), and 1 U is defined as the amount of enzyme needed for catalyzing 1 μmol of substrate per minute under the specified conditions. Triplicate measurements were performed.

2.6. Bisphenol degradation experiments

Degradation experiments of BPA, BPB, BPE, and BPF were performed in 5 mL of 50 mM potassium phosphate buffer (pH = 7) containing 10 mg/L each bisphenol and 0.0035 U/mL PHA-BmTyr biocatalyst in 14 mL test tubes. For BPS degradation, 0.03 U/mL biocatalyst was added into 5 mL of 50 mM potassium phosphate buffer (pH = 7) with 10 mg/L BPS. The reactions were incubated at 30 °C and 225 rpm. At predetermined time points, aliquots of samples were taken and centrifuged immediately to separate the PHA bio-beads from reaction solution for quantification of the bisphenol concentrations in supernatants. The reactions without biocatalyst and reactions using wild-type PHA bio-beads were also included in parallel as abiotic and negative control, respectively. All experiments were performed in triplicates.

Degradation of bisphenols was also tested in real wastewater effluent sample obtained from the local municipal wastewater treatment plant (South Bend, IN). The characteristics of the sample are shown in **Table S3**. The total organic carbon (TOC) was determined using a Shimadzu TOC-L analyzer. The inorganic anions were measured by a Thermo Scientific Dionex ICS-5000 ion chromatography system. The metals were quantified using a Perkin Elmer Optima 8000 inductively coupled plasma-optical emission spectrometry (ICP-OES) system. Prior to use, the effluent was membrane-filtered (0.2 μm) and adjusted to pH = 7.0. The degradation experiments were performed in 5 mL of wastewater effluent spiked with 10 mg/L each bisphenol and 0.0035 U/mL PHA-BmTyr biocatalyst in 14 mL test tubes incubated at 30 °C and 225 rpm. In addition, the degradation in the wastewater effluent spiked with 800 μg/L BPA, BPB or BPE and 0.0007 U/mL biocatalyst was also tested to mimic the environmentally relevant contaminant concentration at μg/L level (Im and Loffler, 2016). For the quality assurance and quality control (QA/QC), abiotic and negative controls were set up, and triplicates were conducted for all experiments. The results were reported as

the average of three replicates with standard errors. The standard calibration curves for quantification of bisphenols were determined by a series of concentrations, ranging from the lowest to the highest ones that covers the estimated or expected concentrations of the analytes. All of the standard calibration curves showed strong linearity with correlation coefficients of > 0.99.

2.7. Molecular docking

The binding modes between BmTyr enzyme and each bisphenol molecule were investigated by molecular docking simulation using Autodock 4.2 (Morris et al., 2009). The detailed method is described in the SI.

2.8. Assessment of toxic effects for degradation products

The recombinant yeast estrogen screening (rYES) bioassay was employed to assess the estrogenic activity of bisphenols and their degradation intermediates and products according to previous research (Balsiger et al., 2010; Zhu and Wei, 2019). Briefly, the human estrogen receptor α (ERα) expression plasmid pG/ER and estrogen-inducible β-galactosidase reporter expression plasmid pUCΔSS-ERE were co-transformed into *Saccharomyces cerevisiae* W303α without the pleiotrophic drug resistance gene (PDR5). The yeast transformant was grown in synthetic complete medium without uracil and tryptophan (SC-UW) overnight at 30 °C and 260 rpm. The cells were transferred in fresh SC-UW at OD_{600} of 0.08 and cultured until OD_{600} reached 0.1. Then, the cells were harvested and exposed to the bisphenol or degradation solutions. After incubation at 30 °C and 260 rpm for 2 h, 100 μL of the exposed yeast culture was mixed with 100 μL of Gal-Screen® Reaction Buffer B (Applied Biosystems, Foster City, CA) in an opaque 96-well microplate for additional 2-h incubation at room temperature. The hormone-induced chemiluminescence was quantified by a Synergy HT microplate reader. 17β-Estradiol was used as the positive control. Triplicates were conducted for all assays.

The acute and chronic toxicity of bisphenol degradation intermediates and products were evaluated using the Ecological Structure Activity Relationships (ECOSAR) program (version 2.0) developed by US EPA. The applicability of ECOSAR has been validated for effective toxicity prediction of chemicals in many studies (Burden et al., 2016; Claeys et al., 2013; Kim et al., 2019). The ECOSAR employs the quantitative structure–activity relationships (QSARs) to calculate the toxicity, in terms of 96 h half lethal concentration (LC_{50}) for fish, 48 h LC_{50} for daphnid, 96 h half effective concentration (EC_{50}) for green algae, and chronic toxicity values (ChV) for all these three organisms.

2.9. Analytical and instrumental methods

A high-performance liquid chromatography (HPLC) system (Agilent 1260 Infinity II) coupled with a diode-array detector (DAD) and an Eclipse XDB-C18 column (Agilent Technologies, Santa Clara, CA) was used to quantify the bisphenol concentration. The mobile phase was methanol and water (v/v = 65:35 for BPA, BPE, and BPF and v/v = 70:30 for BPB) or methanol and water with 0.1% formic acid (v/v = 50:50 for BPS). The flow rate of 1 mL/min was used at 25 °C. The detection wavelength was set as 228 nm (BPA, BPB, BPE, and BPF) or 260 nm (BPS).

The degradation intermediates and products of bisphenols were analyzed by a Dionex Ultimate 3000 Rapid Separation ultraperformance liquid chromatography (UPLC) system equipped with a Bruker MicrOTOF-Q II quadrupole time-of-flight hybrid mass spectrometer (MS). UPLC was performed under an isocratic condition at a flow rate of 1 mL/min with methanol/water (65:35, v/v) through an Eclipse XDB-C18 column. The MS was operated in the negative electrospray ionization (ESI) mode with the following settings: −500 V end plate offset voltage, 2200 V capillary voltage, and nitrogen as both a nebulizer (5 bar) and dry gas (10.0 L/min at 220 °C). The data were collected in the mass range of 100–1600 Da at an acquisition rate of 5000 per second.

3. Results and discussion

3.1. Production and characterization of PHA-BmTyr

The PHA-BmTyr bio-bead biocatalyst was developed by engineering *E. coli* cells with the PHA biogenesis system from *R. eutropha*. There are three key enzymes involved in the PHA biogenesis pathway, including PhaA (a β-kethothiolase), PhaB (an acetoacetyl-CoA reductase), and PhaC (a PHA synthase) encoded by genes *PhaA*, *PhaB*, and *PhaC*, respectively (Sagong et al., 2018). Two recombinant plasmids were constructed accordingly, one harboring the genes *PhaA* and *PhaB* and the other harboring the gene *PhaC* translationally fused with the gene encoding BmTyr for expression of PhaC-BmTyr fusion (**Figure S1**). The engineered *E. coli* co-transformed with these two plasmids would work as a cell factory to produce the PHA-BmTyr biocatalyst as illustrated in Fig. 1**A**. Specifically, under the growth condition with excessive glucose supplement, the glycolysis process in bacterial cells would first convert glucose into acetyl-CoA, a substrate utilized by PhaA to synthesize acetoacetyl-CoA. Subsequently, PhaB would reduce acetoacetyl-CoA into (*R*)-3-hydroxybutyryl-CoA, a monomer precursor for PHA polymers. The PhaB functions with NADPH as the co-factor which could be generated through the conversion of glucose-6-phosphate into ribulose 5-phosphate in the oxidative phase of pentose phosphate pathway. PhaC could further catalyze the polymerization of the monomer precursors into growing hydrophobic PHA chains, which ultimately self-assembled into polymerized PHA bio-beads with PhaC covalently attached on the surface through a thioester bond (Parlane et al., 2017). In this way, the functionalized PHA bio-beads with surface display of the BmTyr enzyme anchored to PhaC would be produced intracellularly in a one-pot process. The PHA-BmTyr biocatalyst can be readily obtained after cell disruption and centrifugation procedures.

After cell cultivation and induction, the PHA bio-beads were formed as confirmed by the Nile red staining (Spiekermann et al., 1999). Nile red is a hydrophobic dye that can bind specifically to PHA with simultaneous strong fluorescence emission. The stained PHA bio-beads in *E. coli* cells could be visualized by fluorescence microscopy, for both the wild-type PHA and engineered PHA-BmTyr, while *E. coli* without PHA production as a negative control had no fluorescence discerned (**Figure S2**). The cells with PHA-BmTyr just showed slightly lower fluorescence intensity than the cells with wild-type PHA, suggesting PhaC could still work properly to synthesize PHA when fused to BmTyr (Fig. 1**B**). The SEM observation showed the isolated PHA bio-beads had mostly round morphology (**Figure S3**). The size distribution of isolated PHA bio-beads in buffer was determined by the DLS with the average hydrodynamic diameter of 295 nm (polydispersity index: 0.109) and 726 nm (polydispersity index: 0.096) for wild-type PHA and PHA-BmTyr bio-beads, respectively (**Figure S4**). The larger size of PHA-BmTyr bio-beads might be attributed to their aggregation in aqueous solution which was also observed for other functionalized PHA bio-beads (Evert et al., 2020; Wong and Rehm, 2018). The SDS-PAGE analysis showed the presence of a major band for both wild-type PHA and PHA-BmTyr (Fig. 1**C**), and the observed molecular weight was consistent with the theoretical molecular weight of PhaC (64.5 kDa) and PhaC-BmTyr fusion (102.1 kDa). As the PhaC and PhaC-BmTyr were expressed with a His tag (**Figure S1**), the proteins were further identified and confirmed using Western blot via specifically probing the His tag (Fig. 1**D**). Furthermore, the enzyme activity of PHA-BmTyr was determined from the formation rate of L-dopachrome through the BmTyr-mediated oxidation of L-DOPA, a model tyrosinase substrate (Faccio et al., 2012). The PHA-BmTyr biocatalyst had an enzyme activity of 112 ± 1 U/g dry weight of PHA bio-beads, while the control wild-type PHA bio-beads showed no detectable activity (Fig. 1**E**). Collectively, these results demonstrated the successful production of PHA bio-beads with display

of functional BmTyr enzyme.

3.2. Biocatalytic degradation of bisphenols by PHA-BmTyr

To evaluate the biocatalytic capability of PHA-BmTyr bio-beads in bisphenol degradation, BPA and its analogues (e.g., BPB, BPE, BPF, and BPS) with frequent occurrences in both engineered and natural water systems were tested as representative contaminants. The results showed that the degradation efficiency for BPA, BPB, BPE, and BPF reached 99.5%, 91.3%, 100%, and 53.5%, respectively after 65 min of reaction, and 100% of BPA, BPB, and BPE and 70% of BPF were degraded within 90 min (Fig. 2**A**). For BPS, prolonged time of 24 h and a higher amount of PHA-BmTyr were needed to achieve a degradation efficiency of 85.3%, suggesting relatively higher recalcitrance of BPS than other bisphenol analogues (**Figure S5**). The low degradability of BPS was reported in previous research (Fang et al., 2020), and the commonly investigated laccase and peroxidase showed no biocatalytic activity to BPS (Beck et al., 2018; Wang et al., 2019b). The strong electron-withdrawing effect of sulfonyl group in BPS which makes the two phenol groups into weak electron donors might decrease its degradability (Fang et al., 2020). There was no bisphenol degradation in the reactions containing the wild-type PHA bio-beads coated with PhaC (**Figure S6**). The results indicated that the PHA-BmTyr bio-beads could efficiently catalyze degradation of various bisphenol analogues, especially BPA, BPB, BPE, and BPF. By comparison, the removal efficiency was reported as ~38%-85% for BPA, ~40% for BPB, and <18% for BPE for AOPs, such as UV/H_2O_2 system, TiO_2-assisted photocatalysis, and UVC photolysis (Luo et al., 2020; Sharma et al., 2015; Wang et al., 2006). Another peroxidase-based biocatalytic method showed limited removal of BPA and BPF (~20%-40%) probably due to enzyme inactivation caused by the added H_2O_2 (Wang et al., 2019b). As BPA, BPB, BPE, and BPF had higher degradation efficiencies and were found to exhibit more potent endocrine-disrupting activity than BPS (Rosenmai et al., 2014; Ruan et al., 2015), we focused on these four bisphenol analogues in following characterization of biocatalytic degradation by PHA-BmTyr in this work.

The bisphenol degradation by the PHA-BmTyr was further characterized by fitting to pseudo-first order kinetics. The relevant parameters shown in **Table S4** indicated the degradation of bisphenols followed pseudo-first order kinetics ($-dC/dt = kC$, where k denotes the pseudo-first order reaction rate coefficient and C denotes the bisphenol concentration at time t). Similar pseudo-first order reactions were also observed in BPA degradation by immobilized peroxidase enzymes (Wang et al., 2019b; Xu et al., 2013). The reaction rate coefficients were 0.0549 min^{-1}, 0.0375 min^{-1}, 0.0648 min^{-1}, and 0.0132 min^{-1} for BPA, BPB, BPE, and BPF, respectively, indicating their degradability by the PHA-BmTyr biocatalyst was in the order of BPE > BPA > BPB > BPF.

To elucidate the different degradability of bisphenols by PHA-BmTyr, the enzyme-substrate interactions at the molecular level were investigated using molecular docking analysis. Molecular docking has been increasingly used to model and predict the interaction between a pollutant-degrading enzyme and its substrate (Liu et al., 2018b). BmTyr has an active site composed of two copper ions, CuA and CuB, which are both coordinated with three histidine residues (**Figure S7**). Based on the catalytic mechanism of BmTyr in transforming phenolic compounds (Deeth and Diedrich, 2010; Goldfeder et al., 2014), the aromatic ring of the substrate would be oriented through a hydrophobic π-π interaction with HIS208 upon entering the active site so that its hydroxyl group could be directed to CuA with a reasonable distance (~2 Å). In this manner, the ortho site of the phenolic ring is positioned towards the proposed oxygen binding site located between CuA and CuB, enabling subsequent electrophilic attack on the phenolic ring to accomplish the catalytic reaction. Accordingly, in the molecular docking simulation, a structure is considered to be correctly docked for catalytic reaction if the substrate benzyl ring is oriented through a hydrophobic π-π interaction with HIS208 and meanwhile its hydroxyl group is directed to CuA for electrophilic attack. The accuracy of molecular docking simulations was verified by docking the *p*-tyrosol molecule into the active site of BmTyr, which had a binding mode highly similar to an experimentally solved X-ray crystallographic structure (**Figure S8**) (Goldfeder et al., 2014). Then, binding complexes between the active site of BmTyr and bisphenols were simulated by molecular docking (**Figure S9**). In accordance with the proposed catalytic mechanism of BmTyr, these docked structures show one of the benzyl rings in all the bisphenol molecules interacts with the HIS208 residue through a hydrophobic π-π stacking and their hydroxyl groups are also directed towards CuA at a reasonable distance (2.28–2.43 Å). Furthermore, the binding energy for the correctly docked structural conformations between the active site of BmTyr and different bisphenols were calculated as −5.31, −5.25, −5.62, and −5.02 kcal/mol for BPA, BPB, BPE, and BPF, respectively. A good relationship was observed between the trend of binding energies for these bisphenols and their reaction rate coefficients when degraded by PHA-BmTyr (Fig. 2**B**). The degradability of a substrate was found to be correlated with binding affinity of enzyme-substrate complex (Bahaman et al., 2021). For instance, the cutinase-mediated degradation activity towards different parabens was dependent on their binding energy to the enzyme, and degradation of four aflatoxins by laccase also had similar results (Liu et al., 2020; Zhu and Wei, 2019). Therefore, the different degradability of bisphenols by PHA-BmTyr could be explained by their varied binding affinities with the active site of BmTyr enzyme,

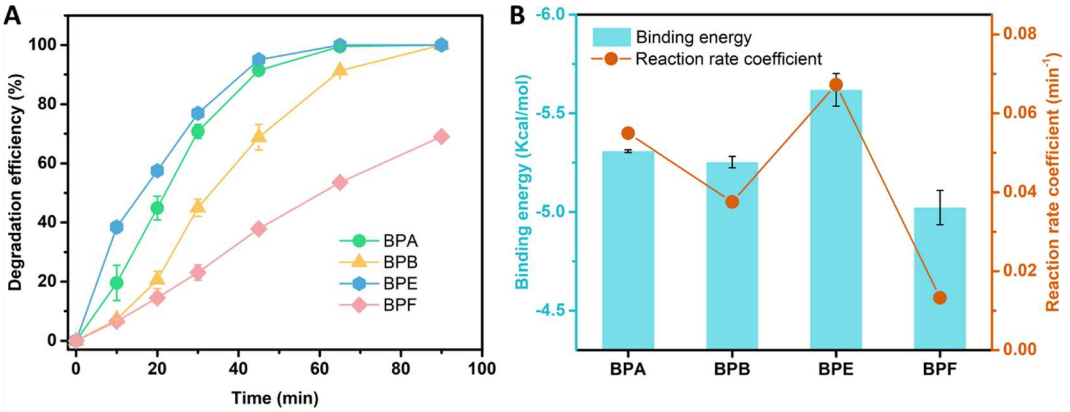

Fig. 2. Degradation of bisphenol compounds mediated by the PHA-BmTyr biocatalyst. (A) Degradation efficiencies of different bisphenols. The initial bisphenol concentration was 10 mg/L. Experiments were conducted in phosphate buffer (pH = 7) with 0.0035 U/mL PHA-BmTyr. The experiments were conducted in triplicates and values represent the mean ± standard error. (B) Correlation of binding energy between the BmTyr enzyme and bisphenols with the degradation kinetics of bisphenols.

leading to different catalytic reactivity.

3.3. Degradation pathway of bisphenols by PHA-BmTyr

To further understand the biocatalytic degradation of bisphenols by PHA-BmTyr, the degradation intermediates and products of BPA, BPB, BPE, and BPF were characterized using UPLC-ESI-TOF-MS. The chromatograms showed that some new peaks appeared, with the size increased in the early stage of degradation process, and then gradually decreased and disappeared as the degradation proceeded (e.g., BPA-P1, BPA-P2, BPB-P1, BPB-P2, BPE-P1, and BPF-P1 in **Figure S10**), indicating formation of degradation intermediates. At the later stage of degradation processes, some new peaks showed up (e.g., BPA-P3, BPA-P4, BPA-P5, BPB-P3, BPE-P2, BPE-P3, BPE-P4, BPF-P2, and BPF-P3 in **Figure S10**), indicating formation of new degradation products from the intermediates. These degradation intermediates and products were identified according to the exact masses determined by the UPLC-ESI-TOF-MS analysis as shown in **Table S5** and **Figures S11-S18**.

Accordingly, the degradation pathway of bisphenols mediated by PHA-BmTyr was proposed (Fig. 3**A**). Tyrosinases are known to catalyze two successive reactions in the presence of molecular oxygen: hydroxylation of monophenols to form *ortho*-diphenols (termed as monophenolase activity) and oxidation of the *ortho*-diphenols into *ortho*-quinones (termed as diphenolase activity) (Claus and Decker, 2006). Specifically, for bisphenols, one of their phenolic groups is initially hydroxylated into an *ortho*-diphenolic group by the monophenolase activity of BmTyr. Then, two reactions might occur: further oxidation at the same phenolic group to form an *ortho*-quinone group through the BmTyr diphenolase activity or hydroxylation at the other phenolic group to have two *ortho*-diphenolic groups through the BmTyr monophenolase activity. Subsequently, either of these two degradation intermediates would be converted into a compound with one *ortho*-diphenolic group and one *ortho*-quinone group by the monophenolase or diphenolase activity, which then would be further oxidized to form a bisquinone molecule containing two *ortho*-quinones. It was reported the quinones from the degradation of other phenolic compounds by tyrosinases were unstable and would non-enzymatically polymerize into insoluble precipitates through complex spontaneous reactions (Ba and Vinoth Kumar, 2017; Kazandjian and Klibanov, 1985; McLarin and Leung, 2020), so we speculate such process might also happen to the bisquinones, ultimately leading to their removal from the water phase by sedimentation or filtration.

3.4. Estrogenic activity and toxicity assessment of bisphenol degradation products

To assess how degradation by PHA-BmTyr works in reducing the endocrine-disrupting effects, the estrogenic activity of BPA, BPB, BPE, and BPF and their degradation products was determined using the rYES bioassay (Balsiger et al., 2010; Petit et al., 1997). The rYES method was validated by using 17β-estradiol, a natural estrogen binding to human ERα, as a positive control. The EC_{50} of 17β-estradiol (5.9×10^{-10} M) obtained from the dose–response curve was in good agreement with other studies (Balsiger et al., 2010; Ruan et al., 2015), confirming the reliability of the rYES bioassay (**Figure S19A**). The EC_{50} of BPA, BPB, BPE, and BPF were determined as 2.39×10^{-6}, 1.3×10^{-6}, 4.67×10^{-6}, and 2.33×10^{-6} M, respectively (**Figure S19B**), well consistent with the previously reported values (Balsiger et al., 2010; Ruan et al., 2015). The

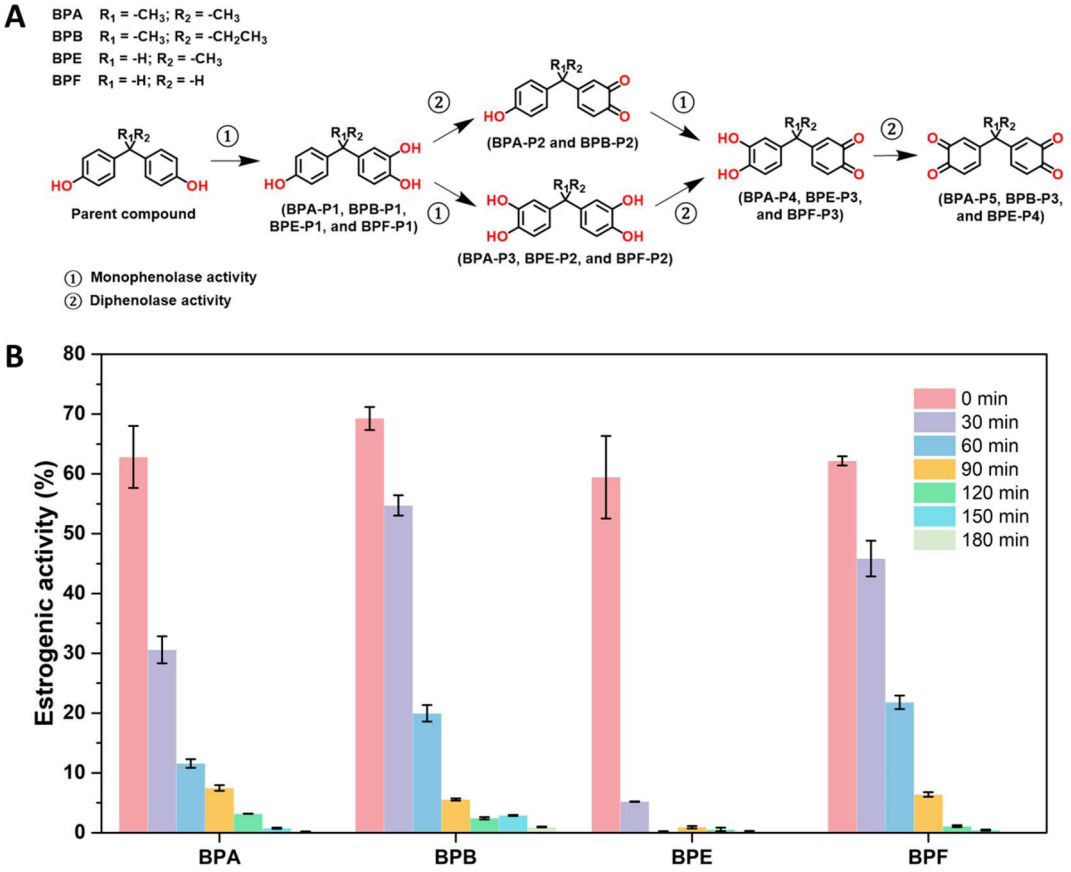

Fig. 3. Degradation pathway and estrogenic activity assay of degradation products. (A) Proposed degradation pathway of bisphenols by the PHA-BmTyr biocatalyst. The identified degradation intermediates and products are shown in parenthesis. (B) Changes in estrogenic activity during the bisphenol degradation by PHA-BmTyr. The estrogenic activity is presented as the percentage response normalized by the maximum activity of 17β-estradiol obtained at the concentration of 1×10^{-7} M. The experiments were conducted in triplicates and values represent the mean ± standard error.

results also confirmed that compared with BPA, the other bisphenol analogue substitutes had similar or stronger estrogenic activity (Ruan et al., 2015). Then, the estrogenic activity of the degradation products was monitored during bisphenol degradation by PHA-BmTyr. The estrogenic activity substantially decreased along the degradation process and was almost undetectable after 180 min (Fig. 3**B**), demonstrating that PHA-BmTyr could effectively mitigate and eliminate the endocrine disrupting effects of parent bisphenols by transforming them into less harmful or harmless products. In comparison, unchanged or even elevated estrogenic activity was observed for treatment of the bisphenols by AOPs, such as ozonation and UV-C photolysis, due to formation of more toxic byproducts (Olmez-Hanci et al., 2015; Plahuta et al., 2014). The biocatalytic approach is advantageous in minimizing the generation of hazardous degradation byproducts because of high specificity of enzyme reactions (Sheldon and Woodley, 2018).

In addition, we evaluated acute and chronic toxicity of the identified degradation intermediates and products towards three model organisms, fish, daphnid, and green algae, by using the ECOSAR program. Almost all of the degradation intermediates and products were predicted to have significantly lower toxicity than their parent compounds, except BPA-P2 and BPB-P2 which showed comparable toxicity to daphnid and green algae relative to parent compounds (**Table S6**). Also, it is noted that there was generally a stepwise trend for toxicity reduction during the bisphenol degradation catalyzed by PHA-BmTyr. For example, the order of the acute and chronic toxicity for BPE and its degradation products was BPE > BPE-P1 > BPE-P2 ≈ BPE-P3 ≫ BPE-P4. These results further corroborated the effective detoxification of bisphenol contaminants during biocatalytic degradation by PHA-BmTyr.

3.5. Storage stability and reusability of PHA-BmTyr

One main purpose of enzyme immobilization is to enable recovery and reuse of the biocatalyst for achieving cost-effective applications. The PHA-BmTyr biocatalyst is essentially functionalized polymeric PHA bio-beads coated with BmTyr enzyme, which could be conveniently recovered from reaction solutions by centrifugation for repeated use. The reusability of PHA-BmTyr was tested in multiple cycles of BPA degradation. Fig. 4**A** shows that PHA-BmTyr could completely degrade BPA in the first 5 cycles of reaction, and the degradation efficiency still remained at 97.3% and 89.3% for the 6th and 7th cycle, respectively. As the biocatalyst was retrieved by centrifugation and then applied to new reaction solutions for each cycle, the high reusability results suggests that there was negligible or limited leakage of the immobilized tyrosinase from the PHA bio-bead surface. The decrease in degradation efficiency after the 6th cycle might be also due to the loss of catalytic activity of immobilized tyrosinase caused by conformational change in the repeated uses. Previous research reported the iron oxide nanoparticle-immobilized tyrosinase displayed 100% in the first 3 cycles but only around 58% after the 7th cycle for degradation of phenol (Abdollahi et al., 2018). The results demonstrated that the PHA-BmTyr biocatalyst could be readily reused with high stability and thus holds great promise for developing cost-effective treatment processes. In respect to the real-world applications, the PHA-BmTyr biocatalyst applied in the wastewater treatment system might be separated from water phase and recovered for re-use through the gravitational sedimentation due to the higher density of PHA bio-beads than that of water. Alternatively, the biocatalyst could be integrated with other solid carriers, such as membranes or hydrogels, which would enable a more convenient recovery.

Maintaining sufficient enzyme activity during storage is another motivation of enzyme immobilization to achieve a proper shelf life for practical applications. The storage stability of the PHA-BmTyr biocatalyst was determined by monitoring the changes in its enzyme activity when stored in 50 mM phosphate buffer (pH = 7) at both 4 °C and room temperature (~22 °C). There was no significant enzyme activity loss for PHA-BmTyr stored at 4 °C for 30 days relative to the initial value, and 86.6% of the initial enzyme activity was still retained even after 30-day storage at room temperature (Fig. 4**B**). In contrast, previous studies reported free tyrosinase could only preserve less than 50% of its initial enzyme activity at 4 °C after 30 days of storage (Labus et al., 2011; Yahşi et al., 2005). The PHA-BmTyr also showed better storage stability in comparison with immobilization of tyrosinases by other conventional methods (Bayramoglu et al., 2013). For example, the tyrosinase chemically cross-linked to Fe_3O_4-NH_2 nanoparticles had only 73.2% of the initial enzyme activity if stored at 4 °C for 30 days, and the remaining enzyme activity of the tyrosinase physically entrapped in alginate or polyacrylamide gel was less than 70% after storage at 10 °C for 21 days (Liu et al., 2018a; Munjal and Sawhney, 2002). These results demonstrated the PHA-BmTyr biocatalyst could maintain superior stability in long-term storage that is ready for practical usage.

3.6. Performance evaluation of PHA-BmTyr under environmentally relevant conditions

First, we investigated the degradation of BPA by PHA-BmTyr at various pH conditions ranging from 5.0 to 8.0 (Fig. 5**A**), as pH is an important water matrix variable that may affect effectiveness of a biocatalyst in contaminant removal. The results show that the degradation

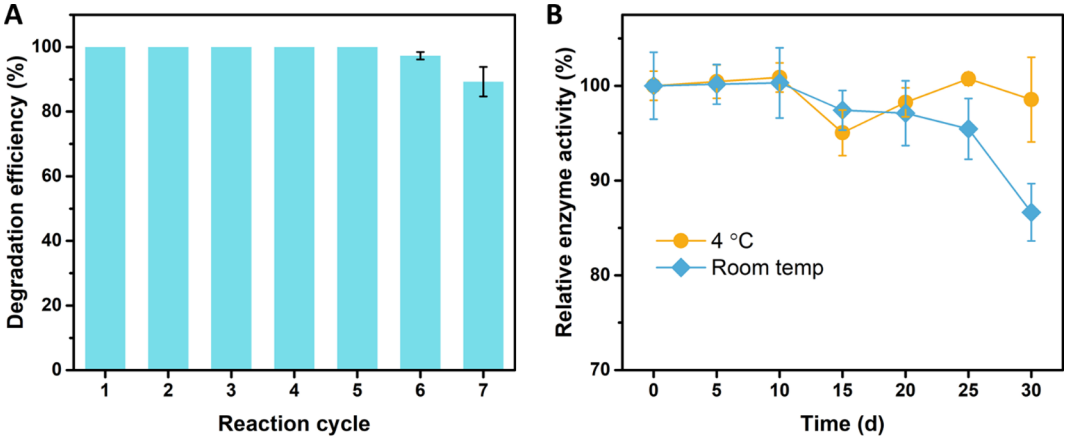

Fig. 4. Reusability and storage stability of the PHA-BmTyr biocatalyst. (A) Reusability of the PHA-BmTyr for bisphenol degradation. The initial BPA concentration was 10 mg/L. Experiments were conducted in phosphate buffer (pH = 7) with 0.025 U/mL PHA-BmTyr at 30 °C for 30 min. (B) Storage stability of the PHA-BmTyr at 4 °C and room temperature. All experiments were conducted in triplicates and values represent the mean ± standard error. Error bars are not visible when smaller than the symbol size.

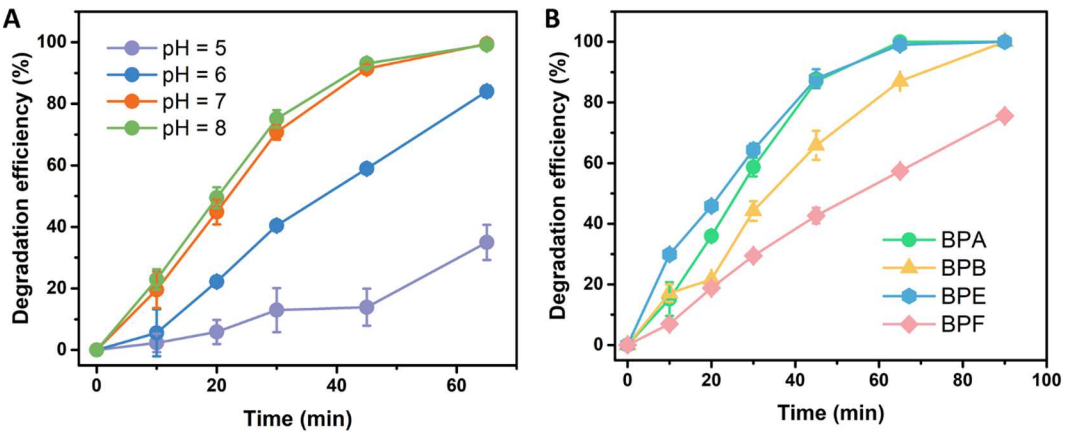

Fig. 5. Performance of the PHA-BmTyr biocatalyst under environmentally relevant conditions. (A) Degradation ability of the PHA-BmTyr at various pH values. The initial BPA concentration was 10 mg/L. Experiments were conducted in acetate buffer (pH = 5) and phosphate buffer (pH = 6, 7, and 8) with 0.0035 U/mL PHA-BmTyr. (B) Degradation of bisphenols by the PHA-BmTyr in secondary wastewater effluent. The initial bisphenol concentration was 10 mg/L. Experiments were conducted in wastewater effluent sample at pH = 7 with 0.0035 U/mL PHA-BmTyr. All experiments were conducted in triplicates and values represent the mean ± standard error. Error bars are not visible when smaller than the symbol size.

efficiency was limited at pH = 5.0 and increased remarkably as pH reached 7.0 and 8.0, suggesting the PHA-BmTyr biocatalyst was most active within the neutral pH range. This is consistent with previous finding that the purified BmTyr exhibited the highest enzyme activity at pH = 7.0 (Shuster and Fishman, 2009). Given the pH of municipal wastewater secondary effluents typically ranges from 6 to 8 (Metcalf et al., 2007), use of the PHA-BmTyr biocatalyst for advanced treatment would minimize the need for pH adjustment and thus reduce the operation cost. In addition, compared with other commonly used enzymes that can degrade the bisphenols under acidic conditions (pH < 6), such as fungal laccases and peroxidases (Strong and Claus, 2011; Wang et al., 2019b), the use of PHA-BmTyr might be preferential when treating the bisphenol-contaminated wastewater with typical pH near neutrality.

Furthermore, the performance of PHA-BmTyr in degrading bisphenols in real wastewater effluent sample from the local South Bend Wastewater Treatment Plant was evaluated. Given the common operation temperature (25–35 °C) for wastewater treatment processes (Morgan-Sagastume and Allen, 2003), the biocatalytic degradation of bisphenols in the real wastewater effluent was performed at 30 °C. Fig. 5**B** shows that PHA-BmTyr maintained high degradation efficiency under the complex wastewater effluent matrix condition, with BPA, BPB, and BPE completely degraded and 75.6% of BPF degraded after 90 min. The bisphenol degradation in wastewater effluent by PHA-BmTyr also followed pseudo-first order kinetics (**Table S7**). When compared with the degradation kinetics in phosphate buffer (**Table S4**), the reaction rate constants were marginally lower in wastewater effluent for BPA, BPB, and BPE, suggesting slight inhibition on the catalytic activity of PHA-BmTyr. However, high degradation efficiencies could still be achieved (Fig. 5**B**), indicating that the PHA-BmTyr biocatalyst has great potential to effectively remove bisphenols in water reclamation. Additionally, a lower concentration of BPA, BPB, and BPE at the level of micrograms per liter was used for degradation in wastewater effluent to mimic the environmentally relevant concentrations (Im and Loffler, 2016). The PHA-BmTyr biocatalyst could also work well in enzymatically removing low-concentration BPA, BPB, and BPE with the degradation efficiency of 98.6% 80.4%, and 100%, respectively after 150 min (**Figure S20**). It is noted that the bisphenols, particularly BPA, BPB, and BPE, were quite recalcitrant in conventional treatment processes with no removal or poor removal rate of < 52% (Wang et al., 2019a; Xue and Kannan, 2019), and ozone-based AOP methods could only degrade ~70% of BPA in wastewater effluent system (Liu et al., 2019). The high degradation efficiency of bisphenols by PHA-BmTyr in wastewater effluent suggested the capability of this new biocatalyst for practical treatment applications. Based on the conditions used in **Figure S20**, the biocatalyst productivity of PHA-BmTyr for BPA, BPB, and BPE could be calculated as 0.050, 0.041, and 0.126 $g_{bisphenol}/(g_{biocatalyst} \cdot h)$, respectively. According to the calculated biocatalyst productivity, for each gram of PHA-BmTyr biocatalyst used, the volume of wastewater containing 800 ug/L BPA, BPB, or BPE that can be treated for removal of these bisphenols could be estimated as 1500, 1230, and 3780 L, respectively. It should be noted that the above estimations and calculations are based on the conditions used in this study. For the practical applications the operating conditions may vary case by case in which scenarios the biocatalyst productivity and treatment volume may need to be re-evaluated accordingly.

4. Conclusions

This study created a novel biocatalyst PHA-BmTyr through genetically immobilizing a bacterial tyrosinase on the surface of PHA biobeads and demonstrated its one-pot in vivo production for effective degradation and detoxification of bisphenols for the first time. Using enzymes for contaminant degradation and environmental remediation has been investigated with most attention on peroxidases and laccases (Routoula and Patwardhan, 2020; Strong and Claus, 2011), while tyrosinases with similar redox catalytic capability remain underexplored so far. One of possible reasons might be the high cost and difficulty in producing purified tyrosinases (Ba and Vinoth Kumar, 2017). The strategy developed in this work could enable simple and economical preparation of a tyrosinase-based biocatalyst with great potential of scaling up for practical use. To scale up the biocatalytic strategy for real-world applications, the important aspects to evaluate and optimize include mass production of the PHA-BmTyr biocatalyst by the recombinant *E. coli* and the design of treatment processes. We envision that the biocatalyst could be used in a suspended form or be further immobilized with membranes for wastewater treatment. Besides, as it is known that tyrosinases have a wide range of substrate, particularly the phenolic compounds, we envision that the PHA-BmTyr biocatalyst could also be used in degradation and detoxification of other phenolic pollutants, such as chlorinated phenols, which merits more investigation in future research. With the versatile and modular PHA display platform used in this study, new biocatalyst could be developed by genetically immobilizing other enzymes of interest onto the PHA bio-beads for biocatalytic remediation of target emerging contaminants.

The PHA-BmTyr biocatalyst has high stability in storage and reuse, and it retains desired catalytic efficiency under environmentally relevant conditions, suggesting its considerable promise in real-world water reclamation applications. The biocatalytic degradation technique is

envisioned to either work independently or collaboratively with other advanced treatment processes as a polishing approach. Compared with engineered whole-cell biocatalysts, the PHA-BmTyr can be regarded as environmentally safe because it is in a cell-free form which prevents the potential risks of genetically modified organisms. Moreover, PHA is a well-known eco-friendly biodegradable biopolymer (Moradali and Rehm, 2020), also minimizing potential environmental impacts of using the PHA-BmTyr biocatalyst even if it is occasionally released into natural environments. Future work can further immobilize this biocatalyst to other carriers such as biogenic hydrogels to achieve enhanced retention and recovery of the PHA-BmTyr biocatalyst in treatment processes.

CRediT authorship contribution statement

Baotong Zhu: Conceptualization, Methodology, Investigation, Writing – original draft. **Na Wei:** Conceptualization, Supervision, Writing – review & editing.

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.

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Appendix A. Supplementary data

Details of the methods for characterization of PHA bio-beads and molecular docking simulation, structures and physicochemical characteristics of bisphenols used in this study (Table S1), the bacterial strains and plasmids used in this study (Table S2), characteristics of the secondary wastewater effluent sample (Table S3), bisphenol degradation kinetic parameters in phosphate buffer (Table S4), degradation intermediates and products of bisphenols identified by UPLC-ESI-TOF-MS (Table S5), acute and chronic toxicity of parent bisphenols and their degradation intermediates and products (Table S6), bisphenol degradation kinetic parameters in secondary wastewater effluent (Table S7), maps of the constructed recombinant plasmids (Figure S1), microscopic observation of the Nile red stained *E. coli* cells (Figure S2), SEM images of the isolated PHA bio-beads (Figure S3), size distribution of PHA bio-beads in buffer solution (Figure S4), degradation of BPS by PHA-BmTyr (Figure S5), degradation of bisphenols by wild-type PHA bio-beads (Figure S6), structure of the BmTyr enzyme (Figure S7), binding conformation between the active site of BmTyr and *p*-tyrosol molecule (Figure S8), binding conformation between the active site of BmTyr and different bisphenol molecules (Figure S9), HPLC chromatograms of bisphenol degradation mediated by the PHA-BmTyr biocatalyst (Figure S10), extracted-ion chromatograms and mass spectrums of the degradation intermediates and products of bisphenols (Figures S11-S18), dose-response curves for estrogenic activity tests (Figure S19), degradation of low-concentration bisphenols by PHA-BmTyr in secondary wastewater effluent (Figure S20). Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2022.107225.

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