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ARTICLE

De novo phenol bioproduction from glucose using biosensor-assisted microbial coculture engineering

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Abstract

Microbial biosynthesis has been extensively adapted for the production of commodity chemicals using renewable feedstocks. This study integrated metabolite biosensors into rationally designed microbial cocultures to achieve high-efficiency bioproduction of phenol from simple carbon substrate glucose. Specifically, two sets of *E. coli*–*E. coli* cocultures were first constructed for accommodation of two independent phenol biosynthesis pathways via 4-hydroxybenzoate (4HB) and tyrosine (TYR), respectively. Biosensor-assisted microbial cell selection mechanisms were subsequently incorporated into the coculture systems to address the insufficient pathway intermediate provision that limited the overall bioproduction. For the 4HB- and TYR-dependent pathways, this approach improved the phenol production by 2.3- and 3.9-fold, respectively, compared to the monoculture controls. Notably, the use of biosensor-assisted cell selection strategy in monocultures resulted in reduced phenol production, highlighting the advantage of coculture engineering for coupling with biosensing. After stepwise optimization, the phenol bioproduction yield of the engineered coculture's reached 0.057 g/g glucose. Furthermore, the coculture biosynthesis was successfully scaled up at both shake flask and bioreactor levels. Overall, the findings of this study demonstrate the outstanding potential of coupling biosensing and modular coculture engineering for advancing microbial biosynthesis of valuable molecules from renewable carbon substrates.

KEYWORDS

biosensor, biosynthesis, *E. coli*, modular coculture engineering, phenol

1 | INTRODUCTION

Phenol is an important commodity chemical with well-recognized industrial values and enormous global market (Bentham & Schulz, 1991). Currently, phenol production relies heavily on the utilization of petrochemicals, which often raises economic, environmental, and sustainability concerns (Schmidt, 2005). As such, biosynthesis of phenol using renewable and inexpensive materials such as biomass feedstocks has received extensive research interest (Bu et al., 2014). Several studies have attempted to establish the phenol bioproduction in engineered microbes. For instance, Wierckx, Ballerstedt, de Bont, and Wery (2005) introduced a heterologous tyrosine–phenol lyase (TPL) to *Pseudomonas putida* S12 to enable the de novo phenol production with a yield of 0.035 g/g glucose. *Pseudomonas taiwanensis* was also utilized for phenol bioproduction, and phenol yields of 0.083 g/g glucose and 0.097 g/g glycerol were reported (Wynands et al., 2018).

Recently, *E. coli* has been adapted as a robust workhorse for phenol biosynthesis, largely due to its amenability to metabolic engineering and bioprocess engineering. Kim, Park, Na, and Lee (2014) developed a

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de novo pathway leading from glucose to phenol via amino acid tyrosine (TYR) in *E. coli*. Assisted by in situ phenol extraction, the engineered strain produced 3.79 g/L phenol with a yield of 0.02 g/g in a biphasic fed-batch bioreactor. Noda, Shirai, Oyama, and Kondo (2016) engineered a platform strain for biosynthesis of chorismate derivatives and achieved 1100 mg/L phenol production from glucose. Miao, Li, Diao, Zhang, and Ma (2015) established a new phenol pathway via the shikimate pathway metabolite chorismate and 4-hydroxybenzoate (4HB). The engineered strain produced 250 mg/L phenol from glucose in shake flask scale production, resulting in a yield of 0.021 g/g glucose. Application of in situ extraction by tributyrin and two-phase fed-batch fermentation further led to 9.51 g/L phenol production using a complex medium containing high concentration of glucose, peptone and yeast extract. In addition, Ren, Yang, Yuan, and Sun (2015) explored the potential of using the third pathway that utilized salicylate as the intermediate and achieved 472 mg/L phenol production using glucose, glycerol and yeast extract as the carbon substrates. Thompson, Machas and Nielsen (2016) compared the phenol biosynthesis using all three pathways and showed that the pathway via salicylate had a higher production yield (35.7 mg/g) than the other two pathways under the analog cultivation conditions. Despite these achievements, further improvement of phenol bioproduction calls for application more sophisticated methodologies.

On the other hand, modularization of biosynthesis pathway in the context of microbial cocultures composed of multiple strains has emerged as a promising approach for microbial biosynthesis and metabolic engineering (Chen, Zhou, Lu, & Zhang, 2019; Jones & Wang, 2018; Zhang & Wang, 2016). In this study, we employed coculture engineering strategies to establish *E. coli*–*E. coli* cocultures to achieve biosynthesis of phenol from glucose. Specifically, two phenol pathways, including the pathways via 4HB and TYR (Figure 1), were accommodated in the designed cocultures consisting of two metabolically engineered *E. coli* strains, respectively. The upstream strains were engineered for provision of pathway intermediates (4HB or TYR), and the downstream strains were employed for converting the intermediates to phenol. The strain-to-strain ratio was flexibly manipulated for pathway balancing and bioproduction optimization.

To enhance the overall bioproduction, metabolite biosensors were engineered and integrated into the designed cocultures. In fact, the application of biosensors in metabolic engineering and synthetic biology has received increasing research interest. For example, several metabolite biosensors have been successfully recruited to distinguish low and high performing cells due to metabolic or genetic heterogeneity (Rugbjerg, Sarup-Lytzen, Nagy, & Sommer, 2018; Snoek et al., 2018; Wang, Cabales, Li, & Zhang, 2019; Yang et al., 2013). In particular, the coupling of the biosensors with selected growth-regulating genes has led to the establishment of robust population quality control or cell selection mechanism by which the growth of the low-performing cells in an isogenic population was inhibited and the overall bioproduction performance was improved (Wang et al., 2019; Xiao, Bowen, Liu, & Zhang, 2016). Yet, the previous achievements using biosensors were mainly made in the context of microbial monocultures, presenting considerable unexplored potentials for applying biosensors in coculture engineering. In this study, we recruited a recently developed microbial

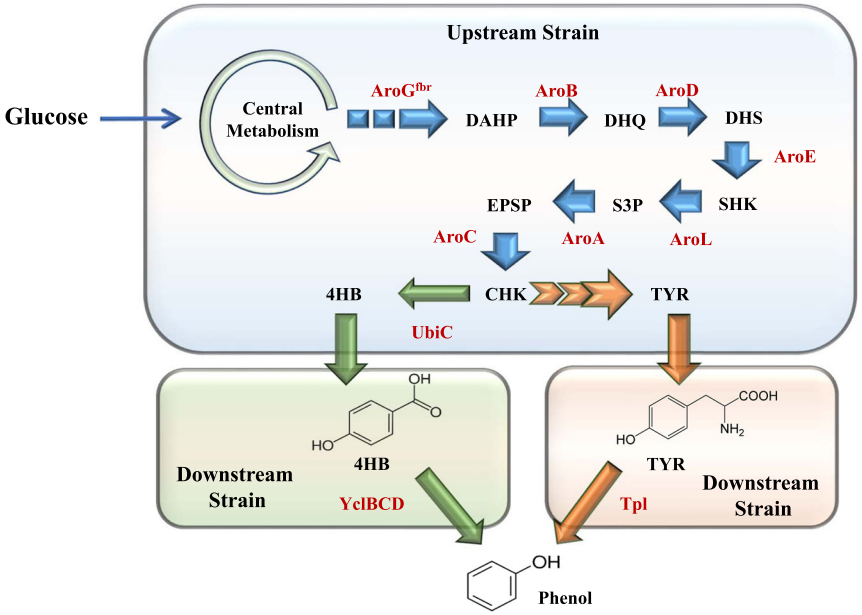

FIGURE 1 The pathways for phenol biosynthesis via 4HB and TYR. AroA, 3-phosphoshikimate 1-carboxyvinyltransferase; AroB, 3-dehydroquinate synthase; AroC, chorismate synthase; AroD, 3-dehydroquinate dehydratase; AroE, shikimate dehydrogenase; AroG^{fbr}, the feedback-resistant (fbr) derivative of the 3-deoxy-7-phosphoheptulonate synthase; AroL, shikimate kinase 2; CHR, chorismate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; EPSP, 5-enolpyruvylshikimate 3-phosphate; S3P, shikimate 3-phosphate; SHK, shikimate; Tpl, tyrosine–phenol lyase; TYR, tyrosine; UbiC, chorismate synthase; YclBCD, 4-hydroxybenzoate decarboxylase; 4HB, 4-hydroxybenzoate [Color figure can be viewed at wileyonlinelibrary.com]

population selection mechanism to address the issue of insufficient pathway intermediate provision that limits overall biosynthesis performance. This new approach opens up new opportunities of engineering microbial cocultures for bioproduction enhancement. Our findings show that the biosensor-assisted cocultures are robust platforms for phenol bioproduction, and the achievement in this study lays a solid foundation for this novel approach's wider application in the broad field of renewable bioresource utilization.

2 | MATERIALS AND METHODS

2.1 | Plasmids and strains construction

All *E. coli* strains as well as plasmids used in this study are summarized in Table S1. Primers used in this study are listed in Table S2. Nucleotide sequences of genes used in this study are listed in Table S3. Detailed information for plasmid construction methods are shown in Supporting Information.

2.2 | Biosensor characterization

The 4HB biosensor-assisted cell selection system is composed of a 4HB-responsive promoter P*pobA* that controls the *tetA* gene expression through the binding with the 4HB-biosensor protein PobR. Strain BHR was constructed by introducing plasmid pRA harboring the *pobR* gene (DiMarco & Ornston, 1994; Jha, Chakraborti, Kern, Fox, & Strauss, 2015) and the P*pobA-tetA* operon into *E. coli* BH2. Strain BH322 was used as a control strain for growth characterization. BHR and BH322 were grown in MY1 medium with varying 4HB concentrations, respectively. The initial OD_{600} was controlled at 0.3 for the growth analysis. The cultures were subjected to cell density analysis by measuring light absorbance at 600 nm after growth at 37 °C under 250 rpm for 18 hr. The tyrosine biosensor-assisted cell selection system is composed of a TYR-responsive promoter P*aroP* that controls the toxin *hipA* gene expression through the binding with the TYR-biosensor protein TyrR. Strain BST was constructed by introducing plasmid pBS9 harboring the P*aroP* promoter and the *hipA* gene into *E. coli* BL21(DE3). Strain TM2 was used as a control strain of BST. Characterization of this system follow the similar method described above. Strain BST and TM2 were grown in the MY3 medium, which had the same composition with MY1 medium except that it contained 2 g/L glucose but no yeast extract.

2.3 | Cultivation condition

Both *E. coli* monocultures and cocultures were cultivated in 3 ml MY1 medium in 37°C at 250 rpm (detailed composition of MY1 medium is shown in Supporting Information). The antibiotics were used in the following concentration: 50 mg/L kanamycin, 34 mg/L chloramphenicol, 100 mg/L ampicillin and 10 mg/L tetracycline. When needed, 0.5 mM IPTG was supplemented to the medium to induce the gene expression under the control of the T7 promoter. For phenol biosynthesis, 2% (vol/vol) overnight LB culture of engineered strains were inoculated in MY1 medium with proper antibiotics and incubated in 37°C for 10 hr to prepare the seed cultures. For monoculture biosynthesis, the cells were then harvested by centrifugation and resuspended in the fresh MY1 medium with an initial OD_{600} of 0.6, followed by 48 hr cultivation at 37°C. For coculture biosynthesis, the upstream and downstream cell cultures were harvested by centrifugation, respectively, and inoculated at desired ratios into 3 ml MY1 medium containing appropriate antibiotics. The initial OD_{600} of the coculture after inoculation was controlled at 0.6. After 48 hr cultivation, the culture samples were taken for HPLC analysis.

For shake flask cultivation, MY2 medium was used for growing the coculture. MY2 medium had the same composition with MY1 medium except that it contained 2 g/L yeast extract to better support the culture growth at the shake flask scale. For fed-batch bioreactor, the overnight MY2 culture of the UY3R and DYR3 strains were added at the ratio of 9:1 to the 2.5 L bioreactor (Eppendorf Bioflo 120) containing 1 L MY2 medium. The initial OD_{600} of the coculture after inoculation was 0.15. Fermentation was carried out at 37 °C with an air flow of 1.5 L/min. Agitation speed was cascaded to maintain the DO level between 3% and 5%. The pH was maintained at 7.0 by automatic addition of 5 M sodium hydroxide and foam formation was suppressed by adding Antifoam B (Silicone Emulsion). Four hundred milliliters of 87.5 g/L glucose solution was fed to the bioreactor at a rate of 0.28 ml/min from 0 hr to 24 hr. For in situ extraction, 100 ml tributyrin was added twice at 12 and 24 hr, respectively.

2.4 | Metabolite and glucose quantification

Quantification of the pathway metabolites was conducted by Agilent 1100 HPLC sets using a method modified a previous report (Wang et al., 2019; detailed methods of HPLC analysis are shown in Supporting Information). Residual sugar in the culture was determined by 3,5-dinitrosalicylic acid (DNS) assay (detailed methods of glucose quantification are shown in Supporting Information; Miller, 1959; Saqib & Whitney, 2011).

2.5 | Determination of the coculture population composition

For the coculture population composition determination, red fluorescence protein (RFP) was used as the marker in the downstream strain DYR3, which allows for differentiation of the coculture strain differentiation using cell flow cytometry. Specifically, 1 ml UY3R:DYR3 coculture samples were collected at different time points during cultivation. The cell pellets were centrifuged and resuspended in 1 ml sterile phosphate buffered saline (PBS) buffer. Such treated samples were diluted 100 times with sterile PBS buffer for flow cytometry analysis. UY3R and DYR3 monoculture samples, collected and treated with the same method, were used as negative and positive controls, respectively. The flow cytometry analysis was performed using a Beckman Coulter CytoFLEX Cytometer with a 488 nm laser and fluorescence channel of 525/40BP. The flow cytometry data were analyzed using Kaluza-Analysis software.

3 | RESULTS AND DISCUSSIONS

3.1 | Construction of the *E. coli*–*E. coli* cocultures for phenol bioproduction via 4HB

To utilize microbial cocultures for de novo phenol bioproduction, two sets of *E. coli*–*E. coli* cocultures were constructed to harbor two different phenol pathways, respectively. As shown in Figure 1, the first set of cocultures were engineered to accommodate the phenol biosynthesis pathway via 4HB (Miao et al., 2015; Thompson, Pugh, Machas, & Nielsen, 2017). In this system, the upstream strain was responsible for producing 4HB from glucose by overexpression of key pathway enzymes, including shikimate dehydrogenase (AroE), shikimate kinase (AroL), 3-phosphoshikimate 1-carboxyvinyltransferase (AroA), chorismate synthase (AroC), chorismate synthase (UbiC; Jiang & Zhang, 2016; Zhang, Pereira, Li, & Stephanopoulos, 2015). The downstream strain was dedicated to converting 4HB to phenol by overexpression of 4-hydroxybenzoate decarboxylase encoded by *yclBCD* genes derived from *E. coli* W strain (Miao et al., 2015).

For this coculture system, we screened a series of *E. coli* strains with different genetic backgrounds, and found that UY2 (*E. coli* BH2 containing pSP2 plasmid contained *aroE*, *aroL*, *aroA*, *aroC*, *ubiC*, and $aroG^{fbr}$ genes) showed the highest conversion from glucose to 4HB (Figure 2a). Therefore, UY2 was used as the baseline upstream strain for the *E. coli*–*E. coli* cocultures in the following experiments. Similarly, a series of *E. coli* strains were tested as the downstream strain for coculture biosynthesis of phenol. Specifically, *E. coli* BL21(DE3), XL10-Gold and BH2 were transformed with plasmid pYCL encoding 4HB decarboxylase gene to yield strain XLDY1, BLDY1 and DY1, respectively. These strains were individually co-cultivated with UY2 for phenol production evaluation. As shown in Figure 2b, UY2:DY1 showed the highest phenol production of 104 mg/L, whereas UY2:XLDY1 and UY2:BLDY1 only produced 13 and 33 mg/L phenol, respectively. Detailed information for the selection of the coculture upstream and downstream strains are shown in Supporting Information.

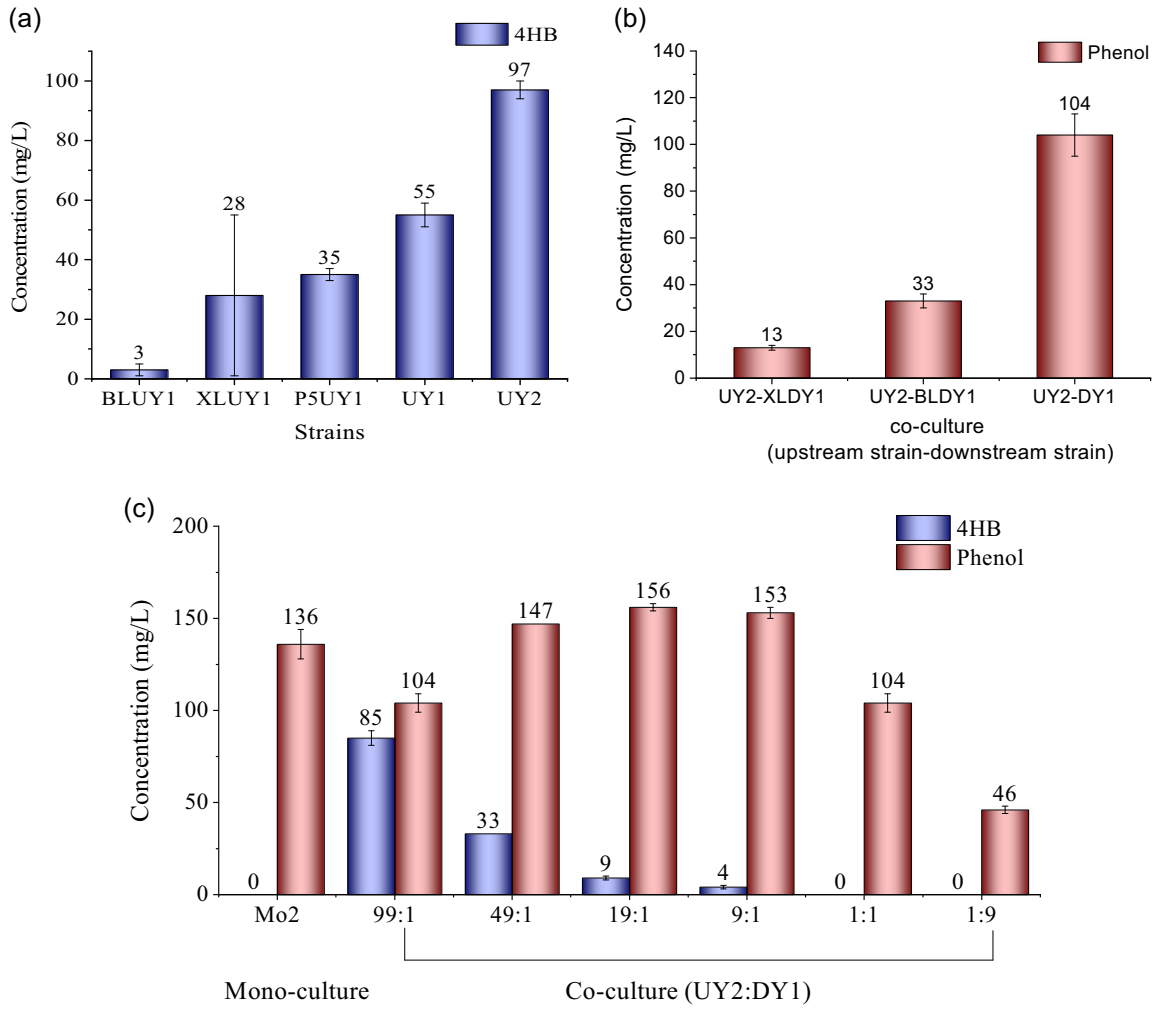

FIGURE 2 Selection of the coculture upstream and downstream strains for phenol biosynthesis through 4-hydroxybenzoate (4HB) and then optimize the phenol biosynthesis. (a) 4HB production comparison between different upstream strains. (b) Comparison of phenol production between UY2:XLDY1, UY2:BLDY1, UY2:DY1 inoculated at 1:1 ratio. (c) Phenol bioproduction by utilization of the Mo2 monoculture and UY2-DY1 coculture inoculated at different ratios [Color figure can be viewed at wileyonlinelibrary.com]

Next, the UY2:DY1 coculture system was optimized for the phenol biosynthesis. Specifically, UY2 and DY1 were inoculated at different ratios to balance the biosynthetic capabilities of the pathway modules harbored by these strains. As shown in Figure 2c, the highest production of 156 mg/L was achieved when the coculture strains were inoculated at the ratio of 19:1. At this condition, the upstream and downstream strains' biosynthetic capability were balanced, leading to low accumulation of pathway intermediate 4HB. However, this finding indicated that the phenol production was still largely limited by the 4HB provision in the cocultures. Notably, the monoculture of the control strain Mo2 (BH2 containing the whole phenol biosynthetic pathway) produced 136 mg/L phenol under the same conditions.

3.2 | Integrating a biosensor-assisted cell selection system into the cocultures for enhancing phenol bioproduction via 4HB

To address insufficient 4HB provision by the upstream strain, we employed a biosensor-assisted cell selection system for 4HB biosynthesis enhancement. Specifically, a 4HB-responsive promoter derived from the *pobA* gene of *Acinetobacter* sp. ADP 1 was coupled with the tetracycline resistance gene *tetA*. The P*pobA* promoter is capable of upregulating gene expression in the presence of 4HB, which is mediated by a transcription factor PobR cloned into the same *E. coli* strain (DiMarco & Ornston, 1994; Jha et al., 2015). Based on this design, the *tetA* gene expression is activated only at a high 4HB concentration, which subsequently results in the growth inhibition of low-performing cells producing low amounts of 4HB. As a result, the microbial population is dominated the high performing cells, leading to the improvement of the overall 4HB bioproduction. The schematic of this design is illustrated in Figure 3a.

To establish the designed biosensor-assisted cell selection system *in vivo*, plasmid pRA was constructed to carry the *pobR* gene and the P*pobA-tetA* operon (Figure S1a). After the transformation of the plasmid into *E. coli* BHR, the desired biosensing-based growth regulation function of the selection system was characterized. As shown in Figure 3b, the growth of BHR depended upon the concentration of 4HB exogenously added to the medium. At low 4HB concentrations, the *tetA* gene was not sufficiently expressed, resulting in lowered cell density in

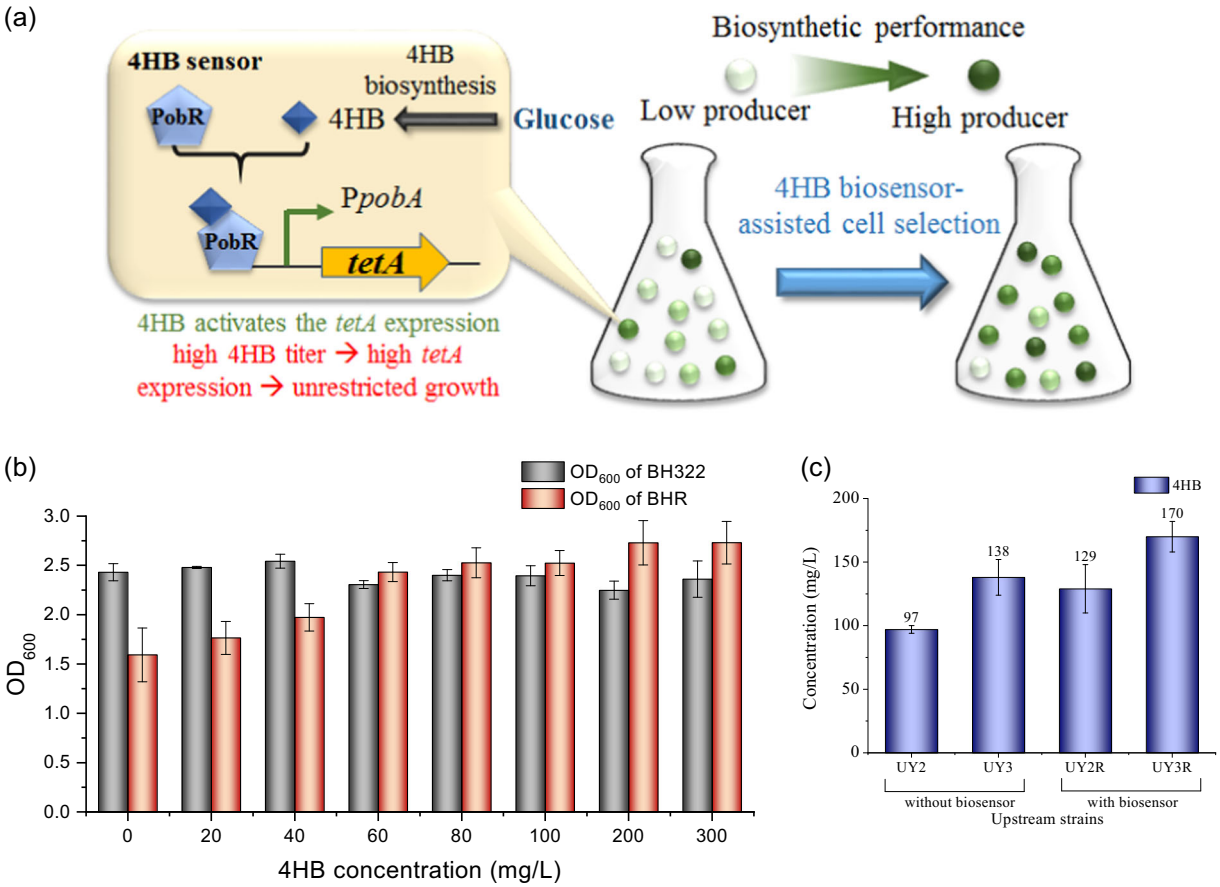

FIGURE 3 Utilization of 4-hydroxybenzoate (4HB) biosensor-assisted cell selection system for enhancing phenol bioproduction. (a) Mechanism of 4HB biosensor-assisted cell selection system for enhancing 4HB biosynthesis performance. High performing cells (4HB over-producers) dominate the population after the selection. (b) Growth of *E. coli* harboring the 4HB biosensor-assisted cell selection system in response to different 4HB concentrations. *E. coli* BH322 and BHR were strains without and with the 4HB biosensor-assisted cell selection system, respectively. (c) 4HB production by the upstream strains without and with 4HB biosensor-assisted cell selection system [Color figure can be viewed at wileyonlinelibrary.com]

the presence of tetracycline. The increase of the exogenous 4HB concentration improved the *tetA* expression and led to increased cell density. In parallel, *E. coli* BH322 was used as a negative control by transforming plasmid pBR322 carrying the *tetA* gene into *E. coli* BH2. The growth of BH322 was not affected by 4HB concentration change. These findings demonstrated that the constructed biosensor-assisted cell selection system indeed had the desired function for regulating cell growth based on the 4HB concentration.

The plasmid pRA was then introduced into the upstream strain UY2 and the resulting strain UY2R was tested for 4HB biosynthesis capability. As shown in Figure 3c, the use of the cell selection system improved 4HB production by 33% (from 97 to 129 mg/L), demonstrating that this strategy indeed enhanced the 4HB bioproduction. When UY2R was co-cultivated with the downstream strain DY1–3 to establish a new coculture for de novo phenol biosynthesis, the phenol bioproduction was significantly improved (Figure 4a). At the optimal inoculation ratio of 19:1 (UY2R: DY1–3), 262 mg/L phenol was produced from 5 g/L glucose, which is 68% higher than the UY2:DY1 coculture that does not use the cell selection system. Notably, it was found that when the upstream strain UY2R was cultivated alone as a monoculture, the metabolic flux toward 4HB formation was improved by 33% compared with the UY2 monoculture. This improvement was much lower than the flux improvement by the UY2R:DY1–3 coculture over the UY2:DY1 coculture (68%). This is because the 4HB concentration in the monoculture reached saturation range of cell selection (Figure 3b) and thus did not generate strong selection pressure for biosynthesis enhancement. However, the 4HB concentration can be considerably reduced through the downstream strain's consumption in the context of a coculture. As a result, the biosensor-assisted cell selection could generate much stronger selection pressure for 4HB biosynthesis and the production improvement for the coculture was much more pronounced than the monoculture. This finding clearly indicates that the power of biosensor-assisted cell selection can be better amplified by coculture engineering.

Interestingly, the introduction of the biosensor-assisted cell selection system into the phenol-producing monoculture control strain reduced the phenol production. In fact, the phenol concentrations for Mo2 and Mo2R (monoculture control strain without and with the 4HB biosensor-assisted cell selection system) were 136 versus 116 mg/L, respectively. This is because the biosensor-assisted cell selection system in the monoculture did not necessarily work to select for cells with high 4HB generation and thus high phenol biosynthesis. Rather, it favored the growth of the cells with low 4HB consumption (high 4HB accumulation), and therefore resulted in reduced phenol biosynthesis. In contrast, this issue was eliminated in the coculture design, as selection of high-producing cells is equivalent to selection of high 4HB accumulating cells of the upstream strain. This finding highlights modular coculture engineering's advantage over monoculture engineering in terms of accommodating biosensors

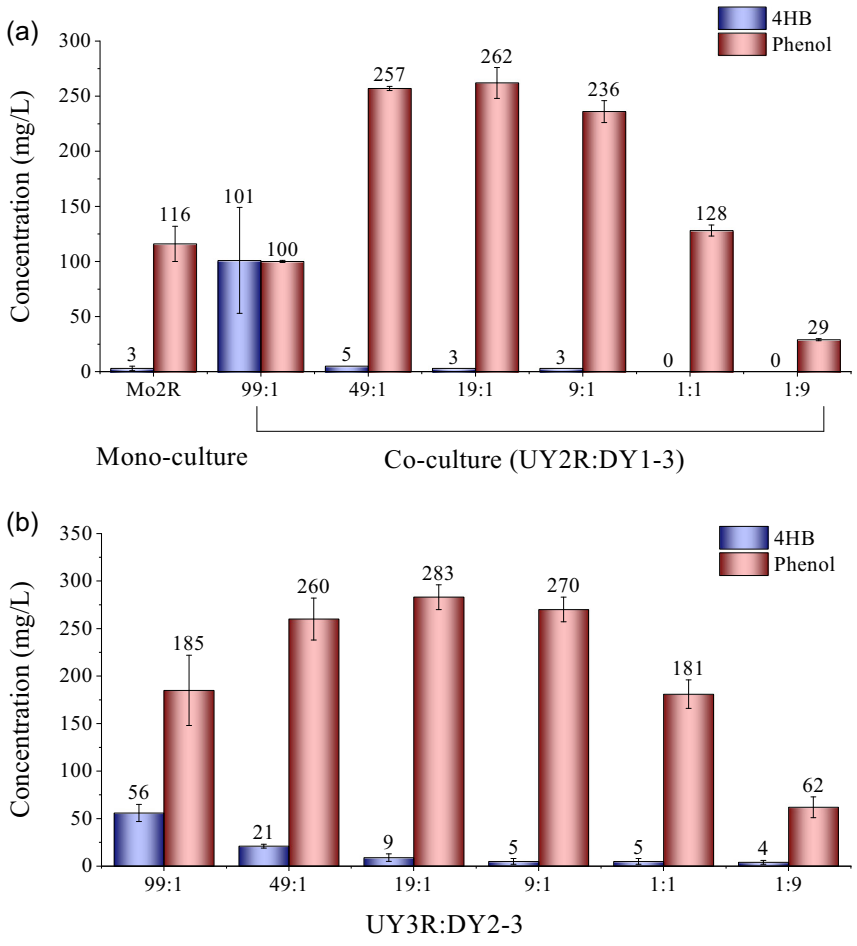

FIGURE 4 Phenol bioproduction by the cocultures with 4-hydroxybenzoate biosensor-assisted cell selection system. (a) Mo2R monoculture and UY2R:DY1–3 coculture; (b) UY3R:DY2–3 coculture [Color figure can be viewed at wileyonlinelibrary.com]

for biosynthesis improvement. In fact, our study showed that, under the same cultivation condition, the UY2R:DY1–3 coculture's phenol production was 2.3-fold higher than the Mo2R monoculture.

Next, the engineered coculture was modified to further strengthen the 4HB supply in the upstream strain. Specifically, two key shikimate pathway enzymes, AroB (3-dehydroquinate synthase) and AroD (3-dehydroquinate dehydratase), were overexpressed in a new upstream strain UY3R. As shown in Figure 3c, 4HB production by UY3R reached 170 mg/L, higher than UY2 and UY2R strains. The improved 4HB supply accordingly resulted in the higher phenol production in the UY3R:DY2–3 coculture. As shown in Figure 4b, at the optimal inoculation ratio of 19:1, the phenol concentration reached 283 mg/L, 8% higher than the UY2R:DY1–3 coculture without the AroB/AroD overexpression. Also, this production was higher than the strategy of over-expressing AroB/AroD without the use of the cell selection system (Figure S2).

After several rounds of engineering efforts (recruitment of proper pathway enzymes, selection of proper upstream and downstream strains, optimization of inoculation ratio, employment of the biosensor-assisted cell selection), our phenol coculture biosynthesis system produced considerably higher concentration of phenol than the starting coculture UY2:XLDY1 (283 vs. 13 mg/L). Also, the coculture biosynthesis performance was substantially higher than the monoculture controls under all tested conditions, demonstrating the effectiveness of the modular coculture engineering strategy for addressing the challenges for biosynthesis optimization. The phenol production yield of 0.057 g/g glucose achieved by the optimized coculture is significantly higher than the previous studies using *E. coli* harboring the 4HB-dependent pathway at the test-tube scale (Miao et al., 2015, Thompson et al., 2016).

According to these results, the integration of the biosensors into the coculture design adds a new dimension to modular coculture engineering. For example, in previous efforts for coculture engineering, balancing the biosynthetic strengths of different pathway modules is mainly achieved by changing the inoculation ratio between the coculture strains. One critical problem of this method is that the strengthening of one particular pathway module unavoidably reduces the strengths of the other module(s). For instance, for a two-strain coculture system, high inoculation ratio for the upstream strain facilitates the improvement of the upstream pathway module, but in the meantime, it reduces the downstream strain's sub-population and the corresponding module's bioconversion power. In contrast, the use of biosensor-assisted cell selection only strengthens the biosynthetic capabilities of the desired pathway module, and does not sacrifice that of the other pathway modules. Therefore, it presents an additional perspective on top of inoculation ratio variation to engineer cocultures for biosynthesis optimization.

3.3 | Construction of biosensor-assisted *E. coli*–*E. coli* cocultures for phenol bioproduction via TYR

In a parallel effort for phenol biosynthesis, another set of *E. coli*–*E. coli* coculture harboring the TYR-dependent pathway was designed and constructed (Figure 1). To this end, *E. coli* strain TPR1 derived from a previously constructed tyrosine over-producer was selected as the upstream strain (Ganesan, Li, Wang, & Zhang, 2017; Santos, 2010; Santos, Xiao, & Stephanopoulos, 2012). For the downstream strain, TPL derived from *P. multocida* (Kim et al., 2014) was overexpressed in *E. coli* YPD1. The production of phenol from 5 g/L glucose was improved by 1.8-fold when biosynthesis was switched from the monoculture strategy to the coculture strategy (Figure 5a).

To improve the TYR availability and the phenol production, a biosensor-assisted cell selection system was also integrated to the coculture. To this end, the *E. coli*'s *aroP* gene's promoter (Pittard, Camakaris, & Yang, 2005) which represses gene expression in the presence of tyrosine facilitated by the transcriptional regulator TyrR bound with tyrosine was recruited. The *E. coli* toxin *hipA* gene (Black, Irwin, & Moyed, 1994; Rotem et al., 2010) was placed under the control of the P*aroP* promoter to regulate the growth of the host strain (Figure S1b). The constructed P*aroP-hipA* operon was introduced into the upstream coculture strain that has an intact chromosomal *tyrR* gene. Based on this design, high concentration of tyrosine in the high performing cells represses the toxic *hipA* gene expression and thus maintains the normal cell growth. In comparison, the low-performing cells' growth is inhibited due to the unrestricted expression of toxic *hipA* gene. As a result, the population of the engineered upstream strain is dominated by high performing cells for enhancing production of tyrosine (Figure 5b). The biosensing-based growth regulation function of the P*aroP-hipA* selection system was characterized, and the results clearly confirmed that this constructed biosensor-assisted cell selection system had the desired TYR-sensing and growth regulation functions (Figure S3).

Next, the TYR biosynthesis capability of the new coculture strain TPS1 harboring the P*aroP-hipA* operon was studied. As shown in Figure S4, TPS1 produced 524 mg/L TYR within 48 hr, which was 285% higher than TPR1 without the cell selection system. It was therefore confirmed that the TYR biosynthesis was successfully enhanced by this strategy. The TPS1 strain was then recruited as a new upstream strain for coculture with YPD1. As shown in Figure 5c, phenol biosynthesis by the TPS1:YPD1 coculture was greatly increased. Compared with the TPR1:YPD1 coculture without the integration of the biosensor-assisted cell selection system (P*aroP-hipA* operon), the new coculture showed higher phenol biosynthesis at most of the tested inoculum ratios. The phenol concentration of 131 mg/L was achieved at the inoculation ratio of 1:4, 66% higher than the optimal production by the TPR1:YPD1 coculture. In comparison, the control monoculture strain MPS1 with the biosensor only produced 34 mg/L phenol under the same condition. The use of the biosensor in the monoculture strain decreased the phenol production via TYR (MPR1 vs. MPS1), which is consistent with the findings of the monoculture production using the 4HB-dependent pathway.

On the other hand, considerable TYR accumulation was observed for the TPS1:YPD1 coculture, indicating that the TPL activity was not sufficient for complete conversion of TYR and thus limited the phenol production in *E. coli*. This result was also observed in a previous study

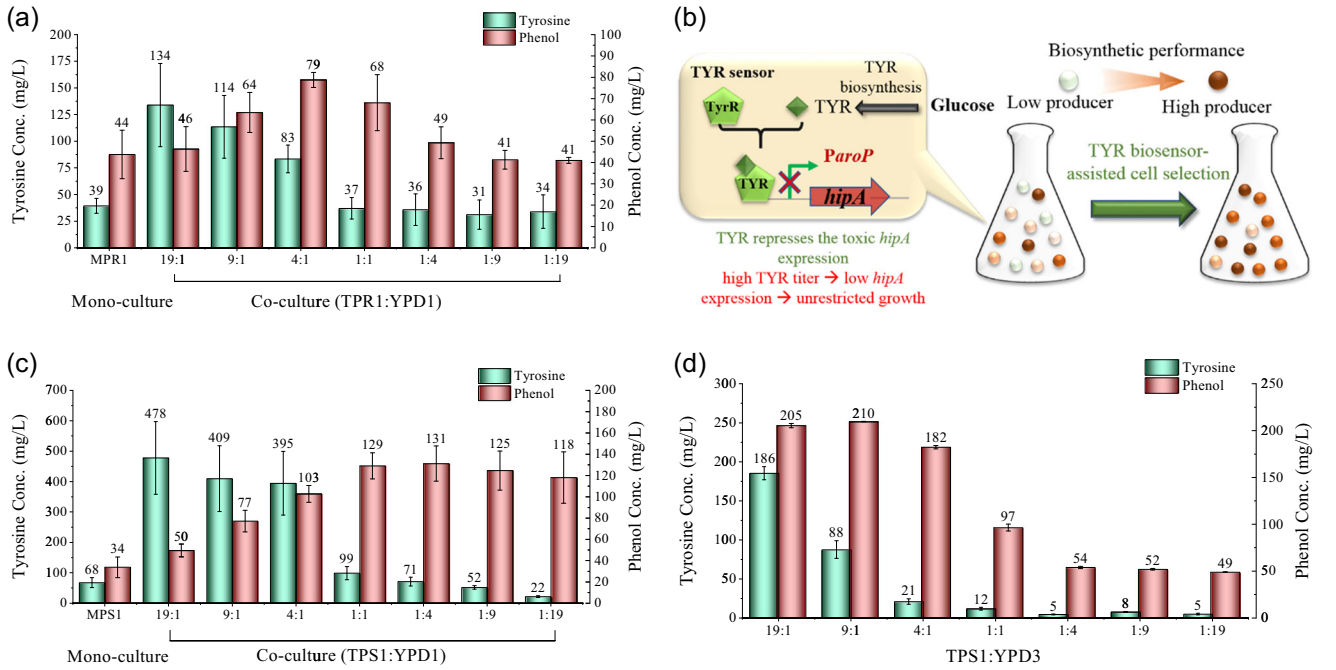

FIGURE 5 Utilization of TYR-biosensor-assisted cell selection system for enhancing phenol bioproduction. (a) Tyrosine and phenol production by the MPR1 monoculture and TPR1:YPD1 coculture inoculated at different ratios. (b) Mechanism of TYR-biosensor-assisted cell selection system for enhancing TYR biosynthesis performance. TYR over-producers dominate the population after the selection. (c) Tyrosine and phenol production by the MPS1 monoculture and TPS1:YPD1 coculture inoculated at different ratios. (d) Tyrosine and phenol production by TPS1:YPD3 coculture with different inoculation ratios. YPD3 contained a strong constitutive P*proC* promoter for the *Tpl* gene expression [Color figure can be viewed at wileyonlinelibrary.com]

using the same TPL enzyme in *E. coli* (Kim et al., 2014). Notably, the optimal inoculation ratio before and after the integration of the biosensor-assisted cell selection system varied from 4:1 to 1:4. This was because the TYR biosynthesis capability of the upstream strain was dramatically improved (Figure S4), which significantly reduced the need of high inoculum of this strain. As a result, the optimal inoculation ratio shifted to 1:4 to allocate more metabolic resources to the downstream strain for pathway balancing.

To further improve the phenol production, the biosensor-assisted coculture underwent additional modifications. The use of the constitutive promoter, *Zymomonas mobilis* pyruvate decarboxylase promoter P*pdc* (Conway, Osman, Konnan, Hoffmann, & Ingram, 1987), for pathway gene expression was not shown to improve the phenol production by the coculture (Figure S5). In addition, the use of another synthetic constitutive promoter P*proC* (Davis, Rubin, & Sauer, 2010), boosted the phenol production. As shown in Figure 5d, at the optimal inoculation ratio of 9:1, the TPS1:YPD3 coculture using the P*proC* promoter produced 210 mg/L phenol, 60% higher than the TPS1:YPD1 coculture.

For both 4HB- and TYR-dependent pathways, the use of the biosensor-assisted cell selection systems improved the overall phenol production by 2.3- and 3.9-fold, respectively, compared to the monocultures engineered using the same cell selection systems. These results clearly demonstrate the strong application potential of biosensor-assisted cell selection system in the context of coculture engineering. It is noteworthy that the use of pathway intermediate biosensors did not improve the phenol production in monocultures, largely because this strategy could select for cells with low pathway intermediate conversion but not necessarily high phenol production. The use of this strategy in the context cocultures, however, eliminated the issues by physical segregation of the pathway intermediate provision and conversion modules. Hence, the use of microbial cocultures offers a unique platform for exploiting the power of biosensor-assisted cell selection. Nonetheless, after stepwise construction and optimization of the coculture systems, we achieved high production yields (0.057 g/g for 4HB-dependent pathway, and 0.042 g/g for TYR-dependent pathway) using glucose as the carbon substrate by the test-tube scale cultivation.

3.4 | Scaling up the engineered coculture system for phenol bioproduction

After construction and modification of the coculture systems, their biosynthesis performance at larger scales was investigated. To this end, the UY3R:DY2–3 coculture harboring the 4HB-dependent pathway was selected as the phenol bioproduction system. To distinguish the coculture strains in the same culture and obtain the dynamic profile of coculture population composition, the downstream strain was labeled with a RFP by expressing the encoding

DsRed gene. The resulting downstream strain DYR3 was cocultured with UY3R for the scale-up experiments.

We first analyzed the coculture biosynthesis performance in shake flask. At this scale, the optimal inoculation ratio was shown to be 9:1 (Figure S6). The growth and biosynthesis time profile of the UY3R:DY2–3 coculture inoculated at this ratio was shown in Figure 6a. The cell density increased rapidly in the first 12 hr, and then stabilized at around 3.0 OD_{600}. Interestingly, the percentage of the downstream strain DYR3 changed from 10% to 28% in the first 12 hr, followed by gradual increase to 58% towards the end of the cultivation. On the other hand, as shown in Figure 6b, the phenol production was mainly accomplished in the first 24 hr and stabilized at 304 mg/L after 24 hr. No 4HB accumulation was observed beyond 24 hr. The bioproduction yield reached 0.061 g/g glucose at the end of cultivation.

Next, we adapted bioprocessing engineering techniques to cultivate the coculture in a 2 L bioreactor. Specifically, a fed-batch bioreactor process was developed for growing the coculture through gradual addition of glucose substrate. Since high concentration of phenol has been found to be toxic to *E. coli* (Table S4; Kim et al., 2014), we implemented an in situ extraction strategy using tributyrin to remove phenol from the aqueous cell culture. To minimize the impact of organic extractant on the cell growth, 100 ml tributyrin was added at 12 and 24 hr, respectively.

As shown in Figure 6c, the cell density of the UY3R:DYR3 coculture in the bioreactor gradually increased after inoculation and plateaued at around 9.0 OD_{600} after 36 hr. Interestingly, the FACS analysis showed the growth of UY3R had consistent advantage over DYR3 at the first 36 hr. After the glucose concentration declined to zero, the DYR3 percentage in the whole population increased to 34%. The phenol concentration in the aqueous phase reached 263 mg/L at 12 hr (Figure 6d). After tributyrin was added to the culture at 12 and 24 hr, the aqueous phenol concentration dropped down to less than 200 mg/L for the rest of the cultivation. Phenol product was enriched in tributyrin and increased up to 8.37 g/L toward the end of cultivation. Combining the phenol accumulation in the aqueous and organic phase, the total amount of phenol produced by the coculture showed steady increase throughout the cultivation. The final production yield, defined as the mass of phenol product over the mass of consumed glucose, stabilized at 0.053 g/g glucose at the end

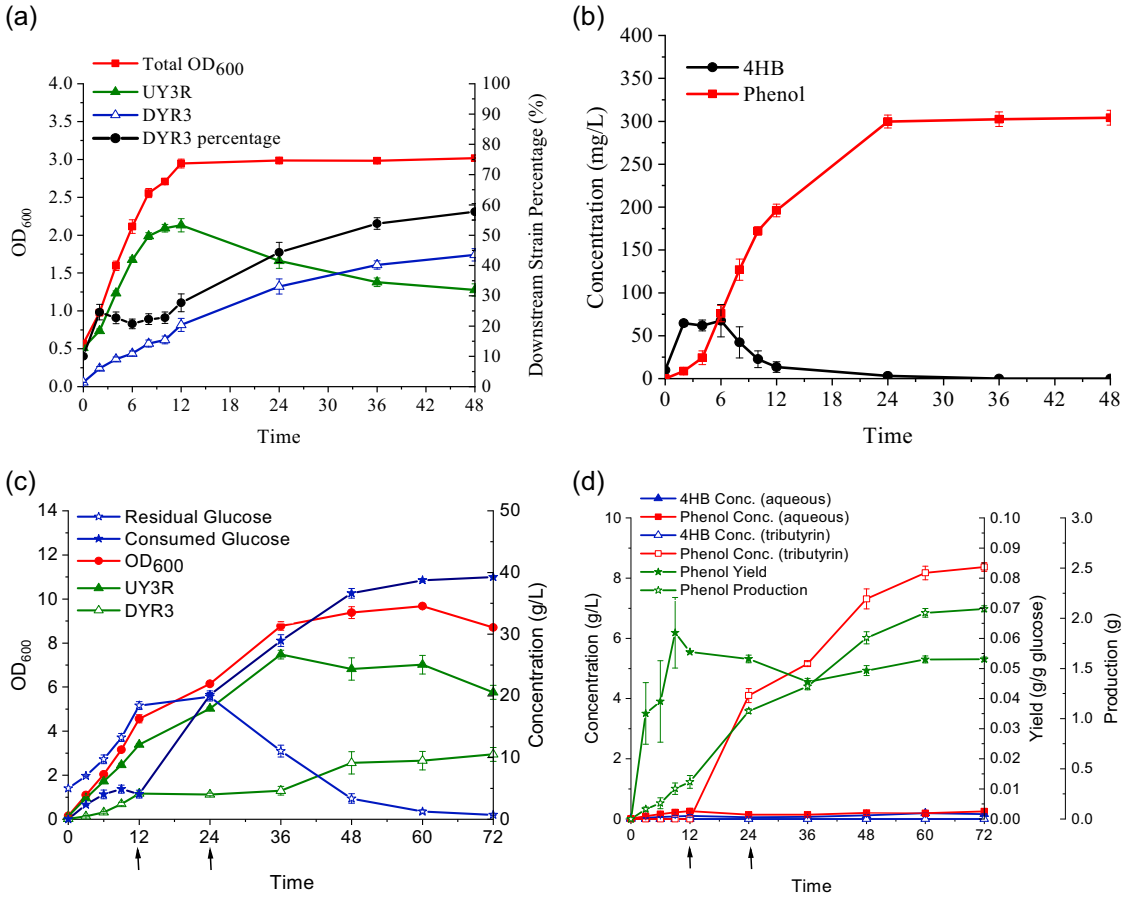

FIGURE 6 Phenol bioproduction by the UY3R:DYR3 coculture cultivated in shake flask (a,b) and fed-batch bioreactor (c,d). (a) Profiles of coculture growth in shake flask. (b) 4-Hydroxybenzoate (4HB) accumulation and phenol production in shake flask. (c) Profiles of coculture growth and glucose consumption by biphasic fed-batch fermentation. (d) 4HB accumulation and phenol production by biphasic fed-batch fermentation. As indicated by the arrows on the figure, 100 ml tributyrin was added at 12 and 24 hr, respectively [Color figure can be viewed at wileyonlinelibrary.com]

of fermentation. Notably, less than 200 mg/L 4HB concentration was observed in the aqueous phase during the whole cultivation process, indicating that 4HB conversion was efficient in the coculture system.

Overall, our findings showed that the use of the biphasic fed-batch bioreactor techniques enabled the scale-up of the coculture system and showed high phenol production performance using renewable sugar glucose as the carbon source. Importantly, we found that the engineered coculture was able to maintain a reasonable strain-to-strain ratio without domination of the entire population by a particular strain. Such coexistence of the coculture strains provided important evidence for microbial coculture system's stability at large scale cultivation.

4 | CONCLUSIONS

This study established two independent coculture systems for bioproduction of phenol via 4HB and TYR, respectively. Biosensor-assisted cell selection systems were integrated into the cocultures as a novel strategy to enhance the pathway intermediates supply and improve of overall phenol production. After stepwise construction and optimization of the coculture systems, we achieved high production yields (0.057 g/g for 4HB-dependent pathway, and 0.042 g/g for TYR-dependent pathway) at the test-tube scale cultivation. Finally, the scale-up study demonstrated the scalability of the engineered microbial cocultures and generated important knowledge for the use of other biosensor-assisted microbial cocultures in the future.

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CONFLICTS OF INTEREST

The authors declare no financial or commercial conflict of interest.

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