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RESEARCH ARTICLE

Minor Phenolic Compounds in Detoxified Lignocellulosic Hydrolysates Are the Determinant Factor on Cell Growth and Metabolic Activity of *Escherichia coli*

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ABSTRACT

Phenolic compounds from lignin degradation in harsh pretreatment of lignocellulose persistently exist at low concentrations in hydrolysates even after standard detoxification and enzymatic hydrolysis. This study found that minor phenolic compounds in dry acid-pretreated and biodetoxified wheat straw hydrolysates were the key inhibitors of *Escherichia coli* (*E. coli*). Multiple *E. coli* strains exhibited poor cell growth and metabolic activity in wheat straw hydrolysates with the presence of minor phenolics. However, the cell growth and metabolic activity of *E. coli* strains were significantly recovered with the declining phenolic content. Several recombinant *E. coli* strains successfully fermented 22.17 g/L of ethanol and synthesized 12.04 g/L of cadaverine using wheat straw hydrolysates after the removal of minor phenolics. These findings highlight the critical role of phenolic compounds in the inhibition of *E. coli* strains and provide a foundation for *E. coli* recombinants in biorefinery fermentations for biofuel and biochemical productions.

1 | Introduction

Inhibitory compounds derived from lignocellulose pretreatment include furan aldehydes such as furfural and 5-hydroxymethylfurfural (HMF), weak organic acids such as acetic acid and formic acid, and phenolic compounds such as p-hydroxybenzaldehyde (HBA), vanillin, and syringaldehyde [1–4]. The removal of inhibitors from lignocellulosic hydrolysate is crucially important for efficient subsequent biorefinery fermentations. When furfural, HMF, and acetic acid are removed from lignocellulosic hydrolysates by various detoxification methods, a wide range of microorganisms, such as lactic acid bacteria, *Saccharomyces cerevisiae*, *Corynebacterium glutamicum*, and *Trichosporon cutaneum*, exhibit a robust tolerance to the residual minor phenolic compounds with good cell growth and metabolism [5–8]. However, *Escherichia coli*, one of the most widely used industrial microorganisms, shows stunted growth and metabolic activity in lignocellulosic hydrolysates, although the major inhibitors in the hydrolysates were completely removed [9–12]. The restoration of normal cell growth and metabolic activity of *Escherichia coli* (*E. coli*) in lignocellulosic hydrolysates could enable multiple industrial fermentations using lignocellulosic biomass as feedstock.

Lignin is one of the three major components of lignocellulose with complicated aromatic structures and releases a variety of phenolic derivatives during pretreatment operations, including phenolic aldehydes such as 4-HBA, vanillin, and syringaldehyde, as well as phenolic acids such as p-coumaric acid, vanillic acid, syringic acid, and ferulic acid [13–16]. While various detoxi-

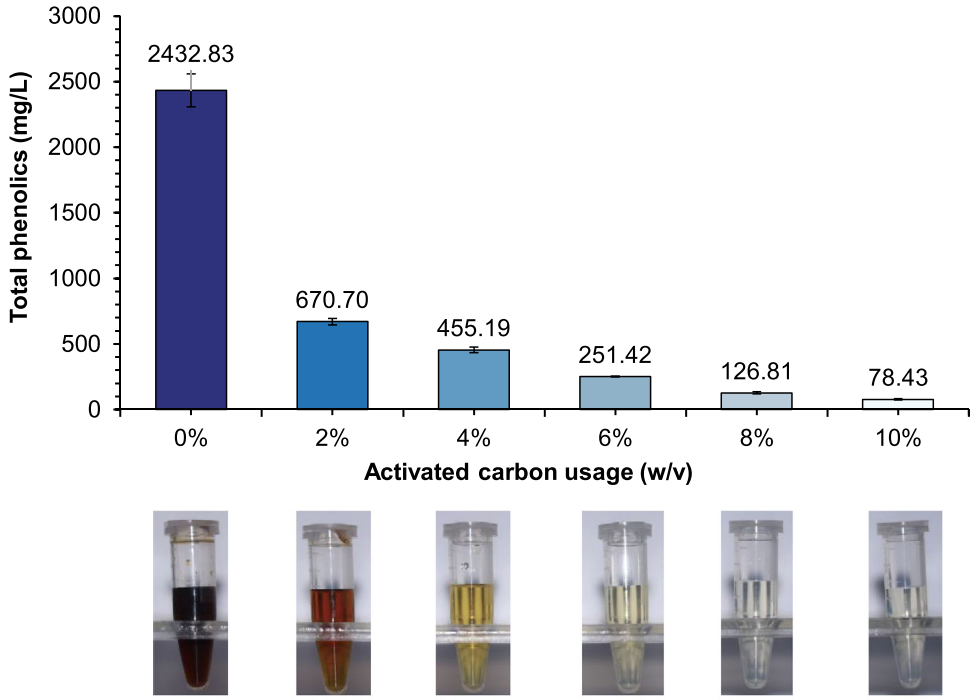

FIGURE 1 | Total phenolic concentrations of wheat straw hydrolysates before and after activated carbon adsorption. The wheat straw hydrolysate was biodetoxified and adsorbed using different amounts of activated carbon powder for 1 h at 60°C. The activated carbon powder was vacuum filtered to obtain the clear wheat straw hydrolysates with varying total phenolic concentrations.

fication treatments such as water washing, over-liming, and biodetoxification efficiently remove furan aldehydes and weak organic acids, the minor phenolic compounds are difficult to remove completely due to their low water solubility and structural diversity [5, 15, 17, 18]. Therefore, the minor phenolic compounds in detoxified lignocellulosic hydrolysates are likely a critical factor for the poor growth and metabolism of *E. coli*.

In this study, several *E. coli* strains were cultured in wheat straw hydrolysates with the complete removal of furfural, HMF, and acetic acid by biodetoxification. The results revealed that the residual minor phenolics in the detoxified wheat straw hydrolysate were the key factor in the restriction of both cell growth and metabolism of *E. coli*. When the phenolics were essentially reduced to a certain low level, engineered *E. coli* strains demonstrated significantly improved fermentability and catalytic activity. The results demonstrated the pivotal role of minor phenolics in inhibiting the cell growth and metabolism of *E. coli* and paved the way for utilizing lignocellulosic feedstocks in *E. coli*-based fermentations.

2 | Materials and Methods

2.1 | Strains and Media

E. coli BL21 and *E. coli* JM109 were purchased from Novagen, and *E. coli* XL1-BLUE from Stratagene. The ethanologenic *E. coli* P81 strain was constructed by integrating the pyruvate decarboxylase gene *pdc* and the ethanol dehydrogenase gene *adhB* from *Zymomonas mobilis* ZM4 into *E. coli* JM109 genome. The cadaverine-producing strain *E. coli* P82 was constructed by cloning the inducible lysine decarboxylase gene *cadA* from *E. coli* K12 into the pET28a(+) plasmid using a seamless cloning kit (Vazyme, Nanjing, Jiangsu, China) and transformed into *E. coli* BL21.

Paecilomyces variotii (*P. variotii*) FN89 was used as a biodetoxification strain. *P. variotii* FN89 was isolated in the previous work [19] and stored in the China General Microbiological Culture Collection (CGMCC), Beijing, China, with the registration number #17665. To prepare the seed culture, the spores were cultured on PDA plates at 37°C for 72 h, then washed out, collected, and inoculated into 100 mL of the medium containing 2 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4$ $7H_2O$, 1 g/L yeast extract, 20 g/L glucose, and 0.5 g/L $CaCl_2$, cultured at 37°C and 300 rpm for 24 h.

2.2 | Enzymes and Reagents

The cellulase used was Cellic CTec3 HS from Novozymes China, Beijing, China. The cellulase protein concentration was measured as 90.1 mg/mL by the Bradford method [20].

Yeast extract and peptone were purchased from Oxoid, Hampshire, UK. The other reagents and chemicals were purchased from Titan Scientific Co., Shanghai, China.

2.3 | Wheat Straw Feedstock and Biorefinery Operations

Wheat straw was collected from Yangqu County, Shanxi Province, China, in 2024. The wheat straw was milled, de-ashed, and then

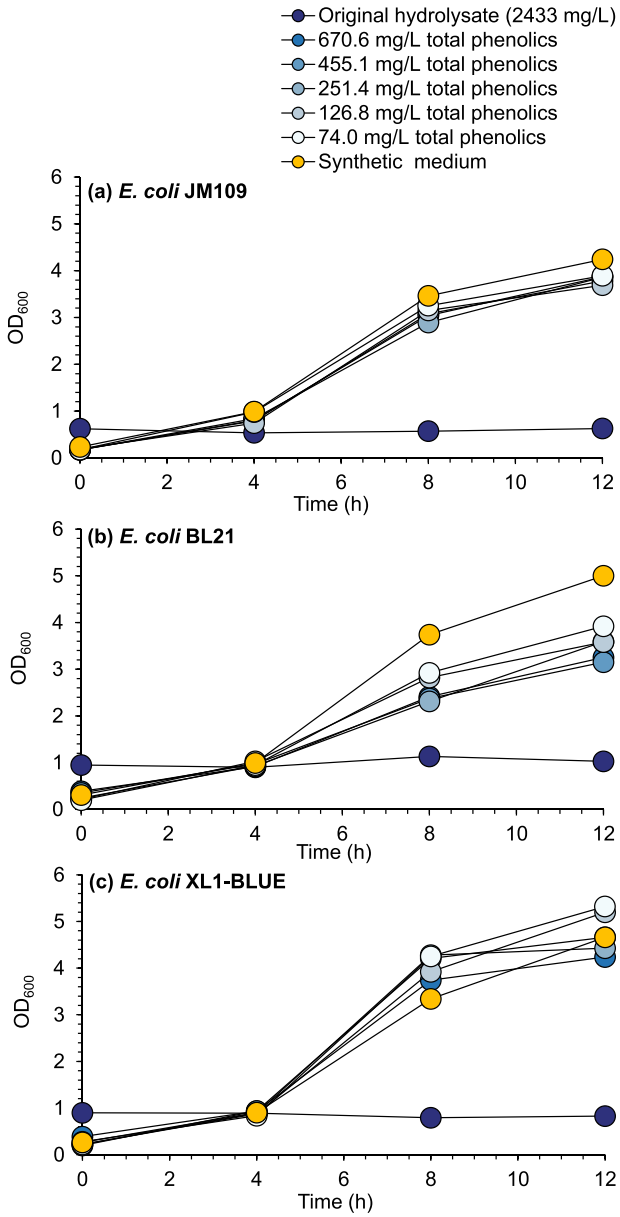

FIGURE 2 | Cell growth of *E. coli* strains in wheat straw hydrolysates with different total phenolic concentrations. (a) *E. coli* JM109; (b) *E. coli* BL21; (c) *E. coli* XL1-blue. The seeds were cultured in LB medium till the OD_{600} was close to 1.0 and then inoculated into wheat straw hydrolysates at 10% (v/v) inoculation. The pH was adjusted to 7.0 with 5 M NaOH solution.

dry acid pretreated in a 10 m^3 industrial pretreatment reactor [19]. The ratio of dry wheat straw to water was 2:1 by weight, and 4 kg of sulfuric acid was used per 100 kg of dry wheat straw [21]. The pretreatment operation was conducted at 175°C for 5 min under well-mixed conditions. No wastewater stream was generated during the pretreatment starting from the feedstock handling to the end of the operations.

After the pretreatment, the wheat straw solids were neutralized using $CaCO_3$ powder to pH 5.5. To prepare 30% (w/v) solid-content wheat straw hydrolysate, 1400 mL deionized water and 2 kg feedstock were added to a 5 L bioreactor. Cellulase was added at a dosage of 4 mg total proteins per gram of solid feedstock, and the enzymatic hydrolysis proceeded at 50°C for 72 h [22]. The main compositions of the hydrolysate after enzymatic hydrolysis completion included 111.5 g/L of glucose, 66.2 g/L of xylose, 2.16 g/L of HMF, 0.43 g/L of furan, and 12.02 g/L of acetic acid.

For biodetoxification, the *P. variotii* FN89 seed broth was inoculated into the wheat straw hydrolysate at an inoculation rate of 10% (v/v) and cultured at 37°C, 750 rpm, and 1 vvm of aeration in a 5 L bioreactor [23]. The detoxification was ended when the pH started to increase rapidly, but before it reached the peak pH value. Then the detoxified hydrolysate was centrifuged at 8000 rpm for 15 min, and the supernatant hydrolysate was collected for the subsequent fermentation.

2.4 | Fermentation and Whole-Cell Catalysis

For seed cultures, the stock vials were grown in LB medium containing 10 g/L NaCl, 10 g/L peptone, and 5 g/L yeast extract at 37°C with shaking at 200 rpm. For fermentation experiments, the seed broth was inoculated at a 10% (v/v) inoculum ratio into the wheat straw hydrolysate containing 10 g/L NaCl, 10 g/L peptone, and 5 g/L yeast extract. The flask fermentations were carried out at 37°C with agitation at 200 rpm for 96 h in 250 mL flasks. The pH was adjusted to pH 7 with 5 M NaOH solution every 8 h.

For whole-cell cadaverine catalysis, the strain was cultured in the wheat straw hydrolysate after declining phenolics until the OD at 600 nm reached 0.6–0.8. Then 0.1 mM IPTG was added at 20°C and incubated for 20 h before the cell harvest by centrifugation at 8000 rpm for 5 min. The collected cells with the expression of *cad*A were transferred into 50 mL of PBS buffer containing 0.1 mM of cofactor PLP (Titan Tech, Shanghai, China) and 60 g/L of L-lysine hydrochloride.

2.5 | Phenolic Removal Operation

Activated carbon powder with a particle size of 200 mesh was purchased from Titan Tech, Shanghai, China, and used to adsorb the phenolic compounds in wheat straw hydrolysate. The hydrolysate was mixed with the activated carbon powder and incubated at 60°C for 1 h with shaking at 200 rpm, and the activated carbon powder was separated from the hydrolysate via vacuum filtration.

2.6 | Analytical Methods

Total phenolic concentrations in wheat straw hydrolysates were determined using the Folin-Ciocalteu method with a commercial assay kit (Grace Biotech, Suzhou, Jiangsu, China).

The spectrophotometer Thermo Fisher BioMate 3S was used for cell density (OD_{600}) measurement.

Glucose, xylose, and ethanol were measured using high-performance liquid chromatography (LC-20AD, Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H column and a refractive index detector (RID-10A). The mobile phase and detection method were 5 mM H_2SO_4, 65°C column temperature,

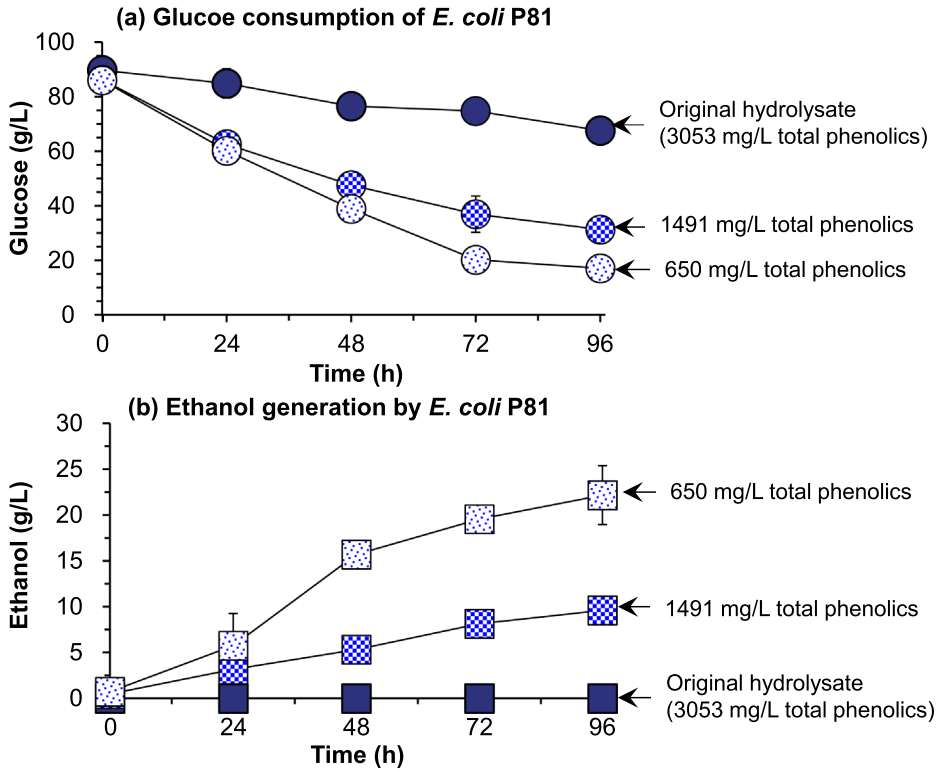

FIGURE 3 | Ethanol fermentation performance of *E. coli* P81 using wheat straw hydrolysates with different phenolic concentrations. (a) Glucose consumption of *E. coli* P81. (b) Ethanol production of *E. coli* P81. Fermentation was conducted in the three wheat straw hydrolysates with different phenolic levels. Each contained 10 g/L NaCl, 10 g/L peptone, and 5 g/L yeast extract. The pH was adjusted to 7.0 with 5 M NaOH solution.

and 0.6 mL/min flow rate sustained for 17 min. The method for detecting inhibitors (furfural, HMF, and acetic acid) employed the same analytical conditions, with an extended time of 55 min.

Cadaverine was measured using a pre-column derivatization method by HPLC [24]. Specifically, 3 μL of DEEMM reagent was added to 50 μL of the sample, followed by sequential addition of 47 μL deionized water, 100 μL of methanol, and 300 μL of borate buffer (50 mM, pH 9.0). The mixture was incubated at 70°C for 2 h, filtered through a 0.22 μm membrane, and subsequently analyzed by high-performance liquid chromatography (LC-20AD, Shimadzu, Kyoto, Japan) using a C18 column (YMC-Park ODS-A, 150 mm × 4.6 mm) maintained at 35°C with a UV detector at 284 nm. The mobile phase included phase A (25 mM sodium acetate, pH 4.8) and phase B (100% acetonitrile). A gradient elution program was applied as follows: 0–2 min, 20%–25% of the phase B; 2–10 min, 25%–70% of the phase B; 10–15 min, 70%–20% of the phase B; 15–20 min, 20% of the phase B. The system was operated at 1 mL/min of the total flow rate.

3 | Results

3.1 | Cell Growth Performance of *E. coli* in Wheat Straw Hydrolysates Before and After Minor Phenolic Removals

The effect of minor phenolic compounds on *E. coli* growth was evaluated using wheat straw hydrolysates containing different levels of phenolic compounds. The furfural and HMF in the wheat straw hydrolysate were completely removed till both were below the HPLC detection lines, and the acetic acid concentration was reduced to ~2 g/L, at which it was no longer considered as an inhibitor [25, 26]. The only significantly identified inhibitors in the wheat straw hydrolysate after biodetoxification were the phenolics, including the representative phenolic aldehydes 4-HBA, vanillin, and syringaldehyde, at a relatively high concentration of 2432.8 mg/L. To establish a phenolic concentration gradient, different activated carbon dosages from 2% to 10% (w/v) were used to absorb the phenolics in wheat straw hydrolysates at 60°C and shaken for 30 min. This treatment generated wheat straw hydrolysates with phenolic concentrations ranging from the original 2430 to 78.4 mg/L (Figure 1).

The cell growth performance of the three *E. coli* strains BL21, JM109, and XL1-BLUE in the wheat straw hydrolysates was evaluated by the optical density at 600 nm (OD_{600}) (Figure 2). The three *E. coli* strains showed almost no cell growth in the original wheat straw hydrolysate (2432.83 mg/L) during the experimental period; however, the cell growth of the *E. coli* strains was significantly improved with the decreasing phenolic concentrations in the wheat straw hydrolysates. In the wheat straw hydrolysate with the lowest total phenolic content (78.43 mg/L), the OD_{600} values of the three *E. coli* strains JM109, BL21, and XL1-Blue reached their maximum of 3.89, 3.92, and 5.32, respectively, very close to that of 4.24, 5.00, and 4.66 in the synthetic medium. The result provided solid evidence that the phenolic compounds in the wheat straw hydrolysates were the key barrier for *E. coli* cell growth, and the cell growth could be quickly recovered once the phenolic compounds were reduced to a low concentration.

3.2 | Ethanol and Cadaverine Fermentations by *E. coli* Recombinants Using Wheat Straw Hydrolysates With Low Phenolic Contents

The existence of minor phenolic compounds in wheat straw hydrolysate had been found to be the determining factor in inhibiting the cell growth of *E. coli* after furfural and HMF were removed. The impact of phenolics on the metabolism of *E. coli* was further investigated on the two typical metabolisms of ethanol fermentation and cadaverine synthesis.

An ethanologenic *E. coli* P81 was constructed by integrating the pyruvate decarboxylase gene *pdc* and the alcohol dehydrogenase gene *adhB* from *Zymomonas mobilis* ZM4 into the *E. coli* JM109 genome. Figure 3 shows that significant changes in glucose consumption and ethanol generation were observed when *E. coli* P81 was inoculated into the wheat straw hydrolysates with different total phenolic concentrations. In the original wheat straw hydrolysate, with furfural and HMF removed by biodetoxification but 3053.5 mg/mL of total phenolic concentration still left in the hydrolysate, *E. coli* P81 consumed glucose slowly, and almost no ethanol was generated. However, when the total phenol concentration was reduced to 1491 and 652 mg/mL by activated carbon adsorption, the glucose consumption rate of *E. coli* P81 was significantly increased, and the ethanol generation reached 9.61 and 22.17 g/L, respectively.

A cadaverine-producing *E. coli* P82 was constructed for cadaverine synthesis using wheat straw hydrolysates as carbohydrates for cell growth or synthesis substrate (Figure 4). The lysine decarboxylase gene *cadA* was cloned into the plasmid pET28a(+) from *E. coli* K12 and transformed into *E. coli* BL21 to obtain the recombinant *E. coli* P82, then inoculated into the wheat straw hydrolysates for cadaverine fermentation. Figure 4a shows that the cell growth of *E. coli* P82 in the original hydrolysate with the high phenolics (3035 mg/L) struggled to reach OD_{600} of 0.6 (the mid-exponential phase) after over 12 h. However, the cell growth of *E. coli* P82 in the low phenolics (650 mg/mL) reached OD_{600} of 0.6 within 4 h. The *E. coli* P82 cells were collected after IPTG induction for expression of the lysine decarboxylase gene *cadA* for 20 h at 20°C and used for cadaverine synthesis. *E. coli* P82 consumed 18.43 g/L of glucose and generated 2.13 g/L of cadaverine, confirming the capacity of *E. coli* to utilize fermentable sugars in the hydrolysate for cadaverine biosynthesis. Figure 4b shows the whole-cell catalysis of the collected *E. coli* P82 cells using lysine as the substrate. The lysine was completely consumed within 8 h, and the cadaverine generation reached 12.04 g/L, confirming that lignocellulosic hydrolysates after phenolic removal were capable of the cell culture for cadaverine catalysis.

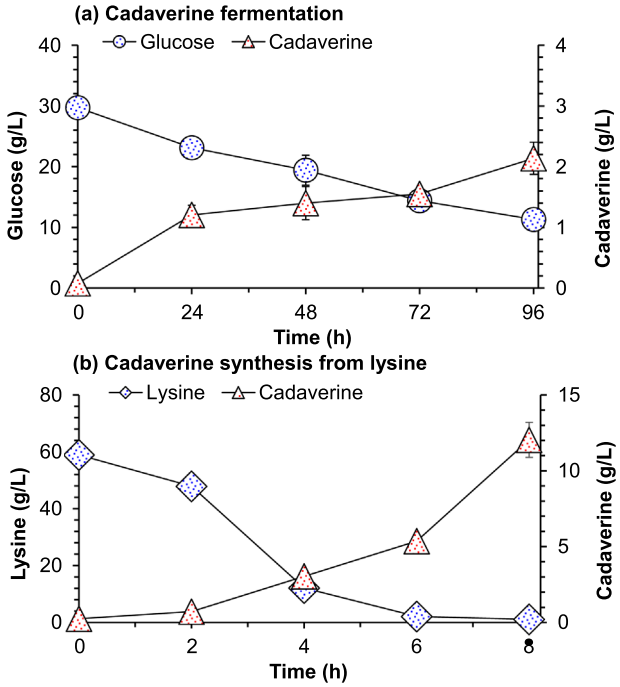

FIGURE 4 | Cadaverine synthesis by *E. coli* P82 using wheat straw hydrolysate with different phenolic contents. (a) Cadaverine fermentation using wheat straw hydrolysate; (b) Cadaverine synthesis using lysine. Cell cultivation was performed in wheat straw hydrolysate with 650 mg/L phenolics and supplemented with 10 g/L NaCl, 10 g/L peptone, and 5 g/L yeast extract. The induced cells were collected and transferred to the fresh hydrolysate for fermentation or to the PBS buffer for whole-cell catalysis with 60 g/L L-lysine hydrochloride and 0.1 mM PLP.

4 | Discussion

Despite the furan aldehydes (furfural and HMF) in lignocellulosic hydrolysates being completely removed and acetic acid being reduced to the minimum level by biological detoxification, the cell growth and metabolism of *E. coli* remain at a negligible level, and the consequent biorefinery applications of *E. coli* were very difficult. This study tried to provide solid evidence for the reasons for the poor growth and metabolic activity of *E. coli*, thus providing a way for biorefinery application of the widely used *E. coli* for the production of various bio-based products.

Phenolic compounds such as 4-HBA, syringaldehyde, syringate, vanillin, ferulic acid, and coniferyl aldehyde are inevitable derivatives from lignin during pretreatment operations, which exert significant inhibitory effects on microorganisms [27, 28]. The stress caused by phenolic compounds on *E. coli* may include various aspects such as disrupting the cell membrane and transport proteins to impede nutrient/waste exchange, inhibiting key metabolic enzymes to block the metabolic network, and inducing oxidative stress, which causes oxidative damage to DNA, proteins, and lipids, ultimately triggering apoptosis [29–31]. Although phenolic compounds are considered harsh inhibitors to microorganisms [27, 32], the regular phenolic levels in lignocellulose hydrolysates after detoxification treatments are not lethal to many fermentation microorganisms used in biorefinery fermentations, such as *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Corynebacterium glutamicum*, and *Gluconobacter oxydans* [33–35]. However, the carefully designed experiments in this study clearly showed that the cell growth and metabolic activities of *E. coli* were very sensitive to even minor existence of phenolics in wheat straw hydrolysates, even after the most harmful inhibitors, such as furfural, HMF, and high-titer acetic acid, were removed by biodetoxification operations. When the phenolics were reduced to a certain level, the cell growth and metabolic activity of *E. coli* restored its fast cell growth and active metabolism. Several recombinant *E. coli* strains successfully fermented 22.17 g/L of

ethanol and synthesized 12.04 g/L of cadaverine using wheat straw hydrolysates under the low phenolic contents.

Unlike furan aldehydes and weak organic acids, phenolic compounds are highly structurally diverse and the least water-soluble. In our previous report [36], a 15% (w/w) solid content wheat straw hydrolysate contained 690 mg/L of total phenolics, with three major inhibitors, HBA (60.21 mg/L), vanillin (79.62 mg/L), and syringaldehyde (30.34 mg/L), contributing only 24.7% of the total phenolics. Therefore, precise component identification of phenolic compounds remains challenging and of limited significance; likewise, physical removal and biological degradation are also rather difficult with current detoxification methods without a considerable loss of fermentable sugars [37]. On the other hand, phenolics are essentially kinds of melanin (black compound) precursors and are easily adsorbed by the commonly porous activated carbon powder [38, 39]. After the phenolics were adsorbed by activated carbon, the cell growth and metabolic activity of *E. coli* were quickly restored, as demonstrated by ethanol fermentation and cadaverine synthesis. However, the use of activated carbon certainly increased the cost and complexity of biorefinery conversions. Metabolic engineering on fermentation microbes could provide an alternatively easy and cost-effective solution.

Previous studies show that the tolerance of *E. coli* to the phenolic components could be improved by adaptive evolution [40, 41], mutagenesis [42], or metabolic modification [43]. As demonstrated by Sierra-Ibarra et al. [17], this enhanced phenolic tolerance enables *E. coli* to achieve an elevated yield in lignocellulosic hydrolysate comparable to that in synthetic media, even at high phenolic concentrations.

Author Contributions

An Wang conducted the *E. coli* recombination. An Wang, Tao Han, and Bin Zhang conducted the phenolic inhibition experiments on *E. coli* and microbial fermentation experiments. Jie Bao designed and conceived the study. An Wang, Tao Han, Bin Zhang, and Jie Bao wrote the manuscript. All authors contributed to the revisions of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The source data supporting the findings of this study are available within the paper.

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