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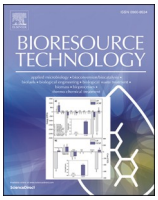

Adaptive evolution of *Paecilomyces variotii* enhanced the biodetoxification of high-titer inhibitors in pretreated lignocellulosic feedstock

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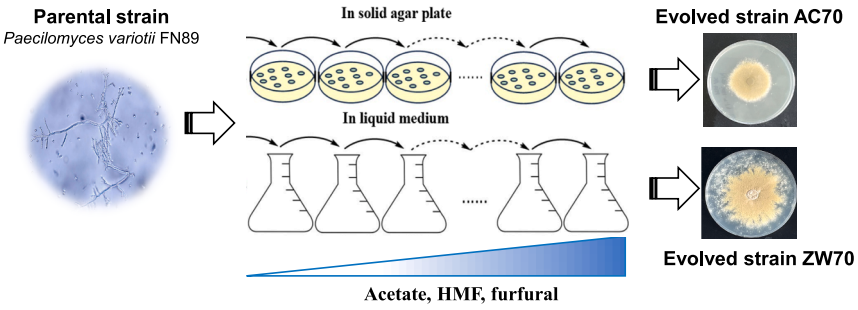
HIGHLIGHTS

- Two ALE methods were designed to boost the biodetoxification of *P. variotii*.
- The evolved strain showed stronger degradation on high titers inhibitors.
- The evolved strains showed upregulation of *adh*, *acs*, *ach1*, and *ackA*.
- Furfural residues were successfully bioconverted to lactic acid.

GRAPHICAL ABSTRACT



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ABSTRACT

High inhibitor concentrations in lignocellulose feedstock negatively affect the degradation rate of biodetoxification strains. This study designed two adaptive laboratory evolutions in solid substrate and liquid medium to boost the biodetoxification capacity of *P. variotii* to high titers of lignocellulose-derived inhibitors, resulting in two evolved strains AC70 and ZW70. The results showed that the evolutionary adaptation in liquid medium could better boost the acetic acid assimilation compared to that on solid substrate. Transcriptional analysis revealed that the evolved strains exhibited a significant upregulation of *adh*, *acs*, *ach1*, and *ackA* directly related to the initial steps of acetate and furan aldehydes metabolisms. ZW70 strain can effectively remove the high concentration inhibitors cocktail from the hydrolysates derived from pretreated wheat straw and furfural residues. The biodetoxified hydrolysates by ZW70 were successfully used for cellulose chiral L-lactic acid production with the titers of ~110 g/L, which were over 20 % higher than that detoxified by parental strain.

1. Introduction

The pretreatment of lignocellulose disrupts the bio-recalcitrance barrier for enzymatic hydrolysis of cellulose and hemicellulose but accompanies the generation of various inhibitory compounds on consequent fermentation microorganisms (Raj et al, 2022a). These inhibitors include furan derivatives such as furfural and 5-hydromethyfurfural (HMF), organic acids such as acetic acid, formic acid, and levulinic acid, as well as phenolic compounds such as vanillin, syringaldehyde, 4-hydroxybenzaldehyde, coniferyl aldehyde, ferulic acid and cinnamic

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acid (Guo et al., 2022; Ran et al., 2014). Although some fermenting strains possess the ability to resist low concentration of inhibitors, the high concentration of inhibitors cocktail could undoubtedly pronounce the inhibition on microbial cell growth and fermentation efficiency resulting in low production performances (Hemansi et al., 2022). Many detoxification methods thus have been developed to remove the inhibitors from the pretreated biomass, including chemical, physical, and biological methods (Raj et al, 2022b).

Biological detoxification (biodetoxification) provides a favorable alternative to general physical or chemical detoxification methods as it has the advantages of mild reaction conditions, low energy consumption, eco-friendly, better selectivity, and fewer toxic byproducts (Singh et al., 2019). The biodetoxification strain tended to convert the phenolic aldehyde inhibitors to corresponding alcohols first, followed by the conversion to acids for ultimate biodegradation (Yi et al., 2019). The isolated *Paecilomyces variotii* FN89 strain in preliminary study can effectively degrade low concentration inhibitors (1.6–1.9 mg/g furfural, 0.6–1.6 mg/g HMF, and 15.2–18.6 mg/g acetic acid) in pretreated lignocellulose (Zhang et al., 2021). The lignocellulosic hydrolysate detoxified by *P. variotii* FN89 has been successfully applied for the production of cellulosic ethanol (Zhang et al., 2021), chiral lactic acid (Zhang et al., 2022a), single cell proteins and microbial lipid (Zhang et al., 2023b).

Generally, high pretreatment severity enhances not only high hydrolysis yield but also inhibitors generation (Zabed et al., 2023). Our modified diluted acid pretreatment method completely eliminated the generation of free wastewater by operating the pretreatment at high solids loading (solid to liquid ratio of 2:1) of raw feedstock (He et al., 2014; Liu et al., 2018). However, all the inhibitors are accumulated into the pretreated feedstock at a very high level, which the contents of furfural, HMF and acetic acid were over 8.0 mg/g, 3.0 mg/g and 30.0 mg/g. Furthermore, the agro-industrial waste feedstocks containing high concentrations of inhibitors, such as furfural residues (Sun et al., 2019), also place stringent requirements on the inhibitor degrading capacity of the biodetoxification strain to facilitate the subsequent bioconversion. Developing robust biodetoxification strains to high concentrations of inhibitors cocktail has become a crucial problem for the efficient valorization of lignocellulosic wastes.

Adaptive laboratory evolution (ALE) is a powerful approach for improving the desired phenotype based on natural selection in a defined environment without an in-depth understanding of the underlying mechanisms in advance (Chen et al., 2024; Bromig and Weuster-Botz, 2023). Adaptive laboratory evolution strategy has been widely implemented to develop robust strains with genetic stability and improved tolerance to lignocellulose-derived inhibitors (Almeida et al., 2023). The strain *Yarrowia lipolytica* with improved tolerance towards ferulic acid (Wang et al., 2021) and vanillic acid (Sha et al., 2023) was obtained through an adaptive evolution strategy. Improved acetic acid tolerance by adaptive evolution was also obtained in *Escherichia coli* (Seong et al., 2020), *Papiliotrema laurentii* UFV-1 (Almeida et al., 2023) and *Halomonas bluephagenesis* (Zhang et al., 2022b).

In general, there're two different media for microbial culture, including solid substrate and submerged liquid medium. In submerged liquid medium, nutrients are dissolved, and oxygen supply is enhanced through agitation with improved oxygen mass transfer and culture uniformity, contributing to better cultivation outcomes (Bakratsas et al., 2021). Solid substrate cultivation facilitates direct interaction between the microbial and the growth substrate under conditions with low water activity (Bentil et al., 2018). In this study, the adaptive laboratory evolutions in both solid substrate and liquid medium were designed to boost the biodetoxification capacity of *P. variotii* to high titers of lignocellulose-derived inhibitors. The comprehensive comparisons were conducted between the evolved and parental strains in terms of morphology, inhibitors degradation capacity and related genes transcriptions. The evolved strains achieved effective removal of high concentrations of acetic acid, HMF and furfural from dry acid pretreated wheat straw and furfural residues hydrolysates. The biodetoxified hydrolysates were subsequently used for cellulose chiral L-lactic acid production (~110 g/L). This study provided an effective strategy for enhancing the tolerance of biodetoxification strains to a high inhibitor environment, providing preliminary information on the molecular mechanisms of target genes involved in the inhibitor metabolism of *P. variotii*.

2. Materials and methods

2.1. Strains and culture

The wild biodetoxification strain *Paecilomyces variotii* FN89 was isolated from dilute acid pretreated corn stover, and stored in China General Microbiological Culture Collection with the assigned number of CGMCC 17665 (Zhang et al., 2021). The adaptive evolution was carried out at 37 °C in both solid substrate and submerged liquid substrate containing lignocellulose-derived inhibitors cocktail. The solid medium was potato-dextrose-agar (PDA) solid gel containing 200 g/L potato infusion, 20 g/L glucose, and 20 g/L agar. The liquid synthetic medium contained 2 g/L of KH_2PO_4, 1 g/L of $(NH_4)_2SO_4$, 1 g/L of $MgSO_4{\cdot}7H_2O$, 1 g/L of yeast extract, 0.5 g/L of $CaCl_2$, and 15 g/L of glucose. The seed culture medium of *P. variotii* was similar to synthetic liquid medium with 20 g/L of glucose.

The strain *Pediococcus acidilactici* ZY271 (CGMCC 13611) was used for cellulosic chiral L-lactic acid production (Qiu et al., 2018). *P. acidilactici* ZY271 was derived from the wild type strain *P. acidilactici* DQ2 by disrupting D-lactic acid and acetic acid synthesis pathways and integrating pentose phosphate pathway. The seed culture medium of *P. acidilactici* ZY271 was Man-Rogosa-Sharp (MRS) medium consisting 20 g/L of glucose, 10 g/L of tryptone, 10 of g/L YE, 5 g/L of sodium acetate, 2 g/L of ammonium citate dibasic, 2.6 of g/L $KH_2PO_4{\cdot}3H_2O$, 0.58 g/L of $MgSO_4{\cdot}7H_2O$, and 0.25 of g/L $MnSO_4{\cdot}H_2O$.

2.2. Raw feedstocks

Raw wheat straw was pretreated by previously established dry acid pretreatment method (He et al., 2014; Liu et al, 2018) on pilot scale in Taiyuan city, Shanxi province, China. The pretreated wheat straw was provided by Cathay Industrial Biotech Inc. (Shanghai, China) in August 2023. The solid residues derived from furfural production (furfural residues) was provided by Jinhe Industrial Co., Ltd (Anhui province, China) in the fall of 2023. The compositions of pretreated wheat straw and furfural residues were determined by NREL protocols (Sluiter et al., 2012).

2.3. Enzymes and reagents

The commercial cellulase enzyme, Cellic CTec 2.0, was purchased from Novozymes (Beijing, China). The filter paper activity, cellobiase activity, and protein content were 203 FPU/ml, 4900 CBU/ml, and 87.3 mg/ml, respectively, based on established protocols reported previously (Adney & Baker, 1996; Bradford, 1976; Ghose, 1987). Yeast extract (LP0021B) and peptone in reagent grade were purchased from Oxoid. Glucose, sulfuric acid, and other chemicals in reagent grade were sourced from Titan Tech Co. (Shanghai, China).

2.4. Adaptive evolution for enhancing the biodetoxification capacity of P. variotii

The adaptive laboratory evolution of *P. variotii* FN89 involved the cultivation on PDA agar plates and in submerged liquid medium with the gradual increasing titers of inhibitors cocktail (Fig. 1). Only the inhibitors of furfural, HMF and acetic acid were focused in this study, because the contents of these inhibitors were much higher than phenolic aldehydes (below 2.5 mg/g DM) in acid pretreated feedstocks and

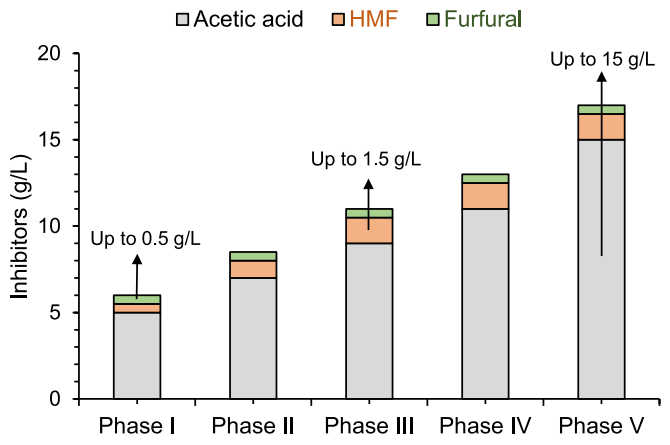

Fig. 1. The concentration of mixed inhibitors for both the adaptive laboratory evolution in solid medium and submerged liquid medium.

furfural residues. *P. variotii* strain had enough capacity to convert these phenolic aldehydes to corresponding less toxic alcohols and acids. The overall adaptive evolution was conducted a total of 70 times of transfers, and divided into five phases including phase I (from 1st to 20th transfer), phase II (from 21st to 35th transfer), phase III (from 36th to 49th transfer), phase IV (from 50th to 60th transfer) and phase V (from 61st to 70th transfer). According to the previous report, the average concentrations of acetic acid, HMF and furfural in hydrolysate can exceed 10 g/L, 1 g/L and 0.1 g/L after high-severity pretreatment (Li et al., 2018). Thus, the concentrations of acetic acid and HMF were increased from 5 g/L and 0.5 g/L to 15 g/L and 1.5 g/L during the adaptive evolution. The concentration of furfural was maintained at 0.5 g/L.

For adaptive laboratory evolution in PDA agar plate, *P. variotii* FN89 was initially cultivated in a PDA agar plate at 37 °C for 3 days, followed by transfer to a fresh PDA agar plate with an initial concentration of 5 g/L of sodium acetate, 1.5 g/L of HMF, and 0.5 g/L of furfural for 3–4 days at constant temperature of 37 °C. After observing the stable cell growth (spores germinated with visible mycelia within 3 days) through 3 sequential passages, the cells were transferred to a fresh PDA agar plate with higher concentrations of inhibitors cocktail. This serial batch transfer was repeated until the 70 transfers.

For adaptive laboratory evolution in synthetic liquid medium, the seed culture of *P. variotii* FN89 was prepared in advance. Briefly, the activation of frozen stock, *P. variotii* FN89, was performed in a PDA agar plate at 37 °C for 3–4 days to produce spores. The collection of spores grown in the plate was carried out by washing the plate using 10 mL of 0.05 % (w/v) Tween 80 solution and then gently scraping the spores using a sterilized cell spreader to get the spore suspension. Then, the suspension was transferred into 100 mL of seed culture medium in a 500 mL flask and incubated for 24 h at 37 °C and 300 rpm. The seed of *P. variotii* FN89 was inoculated into 50 mL of synthetic liquid medium supplemented with 5 g/L sodium acetate, 0.5 g/L HMF, and 0.5 g/L furfural in 250 mL flask at the ratio of 10 % (v/v). The flask was incubated at 37 °C and 300 rpm. The initial pH of the fresh medium supplemented with inhibitors was adjusted to 4.5. The cell broth was serially transferred to 50 mL of fresh liquid media after 72 h cultivation at 10 % (v/v) inoculation ratio. The process in different phases was ended when obvious stable cell density and residual inhibitors were observed. Samples were taken for DCW, acetic acid, HMF, and furfural residual analysis at each transfer. The serial batch transfer was repeated until the 70 transfers.

2.5. Wheat straw and furfural residues hydrolysate preparation

The pretreated wheat straw and furfural residues were subjected to a hydrolysis process in a specially designed 5 L bioreactor equipped with a helical ribbon impeller by fed batch (Zhang et al, 2009). The cellulase enzyme Cellic CTec 2.0 dosage was 4 mg enzyme protein per gram of dry substrate. The final solids loading of the feedstocks was 25 % (w/w), and the pH value was maintained at 5.5 using 25 % (w/w) calcium hydroxide slurry. The hydrolysis conditions for pretreated wheat straw were 50 °C and 200 rpm for 12 h. The hydrolysis conditions for furfural residues were similar to those of wheat straw with the period of 24 h.

2.6. Biodetoxification performance evaluation

The biodetoxification performances of *P. variotii* were evaluated using synthetic liquid medium and lignocellulosic hydrolysate containing high titers of inhibitors cocktail in 3 L bioreactor. The synthetic liquid medium was supplemented with 15 g/L sodium acetate, 1.5 g/L HMF, and 0.5 g/L furfural without pH adjustment, and then the seed of *P. variotii* was inoculated at 10 % (v/v) ratio. The biodetoxification in synthetic liquid medium was conducted at 37 °C, 500 rpm, 1.0 vvm for 48 h. For the biodetoxification of 25 % (w/w) solids loading hydrolysates of pretreated wheat straw and furfural residues, the conditions were 37 °C, 750 rpm, 1.0 vvm for 36 h.

2.7. Fermentability of the biodetoxified hydrolysate for L-lactic acid production

The biodetoxified hydrolysates derived from pretreated wheat straw and furfural residues was subjected to chiral L-lactic acid fermentation in a 3 L bioreactor. The nutrients were added to the hydrolysate including 15 g/L of yeast extract, 10 g/L of tryptone, 2 g/L of ammonium citrate dibasic, and 0.25 g/L of $MnSO_4 \cdot H_2O$ (Zhang et al., 2022a). For the preparation of the seed of *P. acidilactici* ZY271, one vial of glycerol-preserved cells was inoculated into 20 mL of MRS medium in a 100 mL flask and cultured at 42 °C, 150 rpm, for 12 h. Then the broth was transferred into 100 mL of MRS medium in a 500 mL flask and cultured at 42 °C, 150 rpm, for 8 h, as the seed. The glucoamylase was added into MRS medium at a 1 % (w/w) mass ratio to break the polysaccharide links among the cell aggregations, thus preventing cell flocculation. The OD600 values of seed culture are between 3.5 and 5.0. The seed of *P. acidilactici* ZY271 was inoculated at 10 % (v/w) ratio. The fermentation was conducted at 42 °C and 300 rpm for 60 h without aeration.

2.8. RNA extraction and transcriptional analysis

Cell cultivation of the evolved strains and the parental strain *P. variotii* FN89 were carried out in a synthetic medium at 37 °C for 24 h for RNA extraction. The broth was centrifuged at 12,000 rpm to obtain the cells, and total RNA was extracted using Trizol reagent (RNAiso Plus, TAKARA, Otsu, Japan) following the manual protocols. RNA integrity and quantity were verified using a DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Reverse transcription reactions and real-time qPCR (qRT-PCR) were performed, with the 18S rRNA-ITS gene serving as an internal control. The transcription level of the gene was quantified using the formula $2 - \Delta\Delta Ct$. Variations were considered statistically significant at * $P<0.05$, ** $P<0.01$, and *** $P<0.001$. *P. variotii* CBS144490 HYG1 was selected for reference genome, which was preserved in JGI website. Primers are presented in **Table S1** (see supplementary materials), the length of PCR product was 90–200 bp.

2.9. Analytical methods

Glucose, xylose, lactic acid, acetic acid, HMF, and furfural was determined using a Shimadzu HPLC system equipped with a Bio-Rad Aminex HPX-87H column and RID-10A detector. The column temperature was maintained at 65 °C, and 5 mM H_2SO_4 solution served as the mobile phase with a flow rate of 0.6 mL/min. Dry cell weight (DCW) was determined by collecting cell pellets, washing them with distilled water, and drying them at 105 °C overnight until constant weight.

3. Results and discussion

3.1. Adaptive evolution of P. Variotii under high titers of inhibitors cocktail

Adaptive laboratory evolution has been shown to be highly effective in generating microbes with improved growth and enhanced stress tolerance to lignocellulose-derived inhibitors (Menegon et al., 2022). Notably, for filamentous fungi, numerous studies described that the changes in the state (solid or liquid) of culture medium can significantly affect their growth (te Biesebeke et al., 2004), cell structure (De Moraes Borba et al., 2002), metabolites secretion (Kumar et al., 2021), transcriptomics (SantAnna Iwanicki et al., 2023), etc. However, the present adaptive evolution studies paid little attention to comparing the behavioral changes of the strains in different states (solid or liquid) of the substrate. The adaptive evolution of *P. variotii* under high titers of inhibitors cocktail in this study was therefore carried out in both solid substrate and submerged liquid medium (Fig. 2), in order to comprehensively compare the differences of evolved strains in morphology, inhibitors biodegradation capacity, and gene transcriptions.

For the adaptive evolution on solid PDA plates containing inhibitors cocktail (Fig. 2**a**), the cells were transferred to the next stage with higher inhibitor concentrations when growing stable (spores germinated with visible mycelia within three days) in one stage. The colony size of the cells on the PDA gel was changed into smaller ones in the 3rd phase when the inhibitors increased to 9 g/L sodium acetate, 1.5 g/L HMF, and 0.5 g/L furfural. The color of the spores was also changed into a deep yellowish-green from green in the fourth phase when the inhibitors were up to 11 g/L sodium acetate, 1.5 g/L HMF, and 0.5 g/L furfural. The morphology and size of the cell colonies were observed in a stable state without the observable changes after 70 transfers, assuming the cells reached their threshold at the final phase, and the evolved strain was defined as *P. variotii* AC70.

For the adaptive evolution in submerged liquid medium containing inhibitors cocktail (Fig. 2**b**), the initial transfer of *P. variotii* showed short fluctuation in cell growth and residual acetic acid concentrations, but gradually became stable after six to eight consecutive transfers. After the four phases of the evolutionary adaptation process, the strain could maintain stable cell growth at the initial transfer to the submerged medium containing 15 g/L sodium acetate, 1.5 g/L HMF, and 0.5 g/L furfural till final transfer. The evolutionary adaptation was terminated after 70 transfers in 210 days with an indication of a relatively stable stage (~4 g/L dry cell weight) of cell growth, and the residual acetic acid was less than 2.5 g/L. The evolved strain was defined as *P. variotii* ZW70. Additionally, furfural and HMF were preferentially consumed by *P. variotii* during the culture. Afterward, the acetic acid conversion rate was enhanced after the total furfural and HMF achieved almost complete degradation.

3.2. Comparisons of parental and evolved strains in morphology and inhibitors biodegradation

The adaptively evolved strains *P. variotii* AC70 and ZW70 were grown on PDA agar plates with and without adding the inhibitors cocktail including 15 g/L of sodium acetate, 1.5 g/L of HMF, and 0.5 g/L of furfural for morphology characterization (Fig. 3). After three consecutive cultivations and observations, both the evolved strains always showed the smaller colony size transformation compared with the parental FN89 in the existence of inhibitors, perhaps owing to a self-protection mechanism for the evolved strains to cope with higher concentrations of inhibitors cocktail. The evolved strains demonstrated an undulate margin on the colony center as the growth response and produced fewer mycelia, meanwhile, the color of the mycelium of the evolved strains changed into a deeper green color and yellowish-green

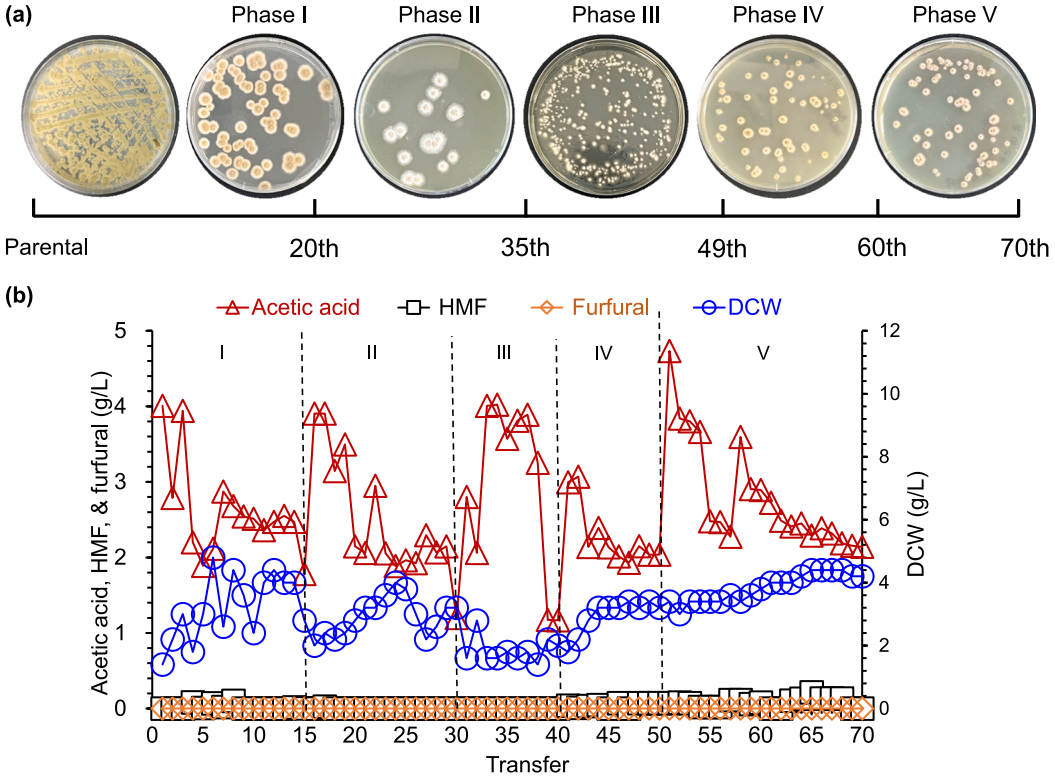

Fig. 2. Adaptive evolution of *P. variotii* FN89 in solid substrate and submerged liquid strate containing high titers of lignocellulose-derived inhibitors. (a) The morphology changes of *P. variotii* FN89 colonies during adaptive laboratory evolution on PDA agar plates. (b) Profiling of dry cell weight (DCW) and concentrations of residual acetic acid, HMF and furfural during adaptive evolution of *P. variotii* in submerged liquid medium. The concentrations of added inhibitors are showed in Fig. 1. The whole adaptive evolution had lasted for 70 generations, equivalent to 253 days on solid substrate and 210 days in submerged liquid medium.

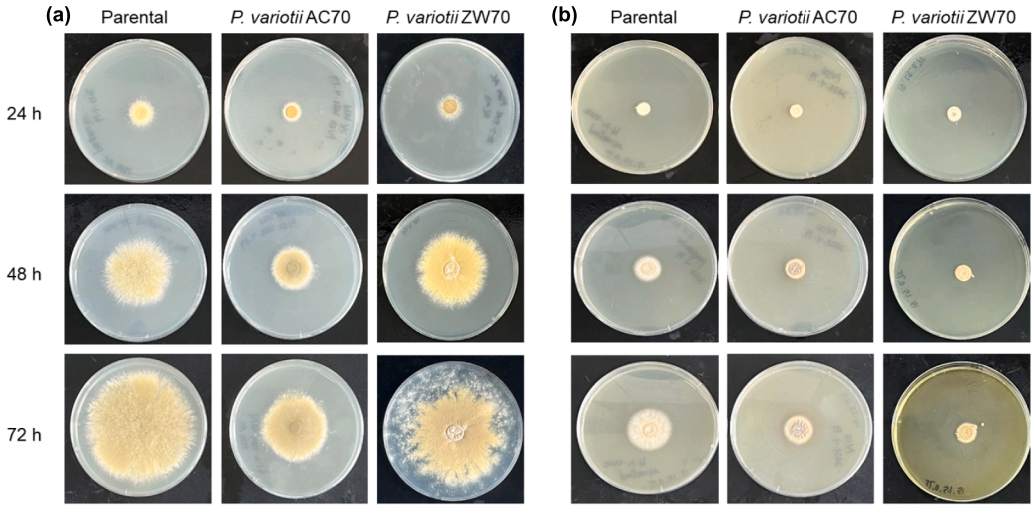

Fig. 3. Morphologies of the adaptively evolved strains *P. variotii* AC70, ZW70 and the parental strain on PDA agar gel. (a) No inhibitors were added to PDA plates. (b) The inhibitors including 15 g/L of sodium acetate, 1.5 g/L of HMF and 0.5 g/L of furfural were added to PDA plates. Culture conditions: 37 °C, 72 h.

color, respectively.

The biodetoxification performances of the evolved and parental strains were evaluated using synthetic liquid medium and lignocellulosic hydrolysate containing high titers of inhibitors cocktail (Fig. 4). For the biodetoxification in synthetic medium containing 15 g/L of sodium acetate, 1.5 g/L of HMF, and 0.5 g/L of furfural, as well as 40 g/L of glucose, 20 g/L of xylose (**Figure S1**) (see supplementary materials), the two adaptively evolved strains showed higher inhibitor conversion rates and better cell growth than the parental with approximately the same sugar consumption. Both the evolved strains showed approximately 1.7–2.0 folds higher degradation of acetic acid than the parental strain after 48 h of cultivation. The evolved strains also exhibited superior cell growth with increased cell density, demonstrating that the DCW was 3.57 and 3.28 times higher than that of the parental strain at 48 h, respectively. The interesting thing is that evolved strain ZW70 demonstrated remarkable removal performances for HMF and furfural, outperforming the evolved strain AC70. The strain ZW70 obtained by evolving in liquid medium showed a relatively better inhibitor degradation capacity than that of the strain AC70 obtained by evolving in solid PDA gel.

Two lignocellulose hydrolysates containing high titers of inhibitors cocktail were selected to further evaluate the biodetoxification performances of the evolved strains, which were derived from pretreated wheat straw (Fig. 4**a-d**) and furfural residues (Fig. 4**e-h**). The raw wheat straw was pretreated under high solids loading (66.7 %, w/w). No free wastewater was generated and all the inhibitors were accumulated into the pretreated biomass (Liu et al., 2018). Moreover, the pretreatment of wheat straw on pilot scale generated higher concentrations of inhibitors cocktail than that on lab scale, perhaps owing to the prolonged heating time in larger reactor (20 L *vs.* 10 m^3). Therefore, the contents of acetic acid, HMF and furfural in wheat straw pretreated on pilot scale reached 30.5 ± 0.2 mg/g, 3.6 ± 0.1 mg/g and 8.9 ± 0.1 mg/g (Table 1), which are 2.0, 6.0, 5.6 folds higher those in wheat straw pretreated on lab scale (Zhang et al., 2021). The hydrolysate using 25 % (w/w) solids loading of pretreated wheat straw contained 9.7 ± 0.2 g/L of acetic acid, 1.4 ± 0.1 g/L of HMF and 1.7 ± 0.1 g/L of furfural (Fig. 4**a-c**). Both the evolved strains showed the accelerated biodegradation rates for the three inhibitors in wheat straw hydrolysate compared with that of the parental strain. All of acetic acid was degraded by the evolved strains within 36 h, while only 57 % of acetic acid was degraded by the parental strain. The significant loss of glucose and xylose during the biodetoxification were not detected (Fig. 4**d**), because *P. variotti* FN89 can consume lignocellulose-derived inhibitors prior to fermentable sugars (Zhang et al., 2021). The concentration of glucose was increased during the biodetoxification due to the simultaneous enzymatic hydrolysis of the residual pretreated wheat straw solids in the slurry by the added commercial cellulase. There were no significant differences in the biodegrading profiles of the inhibitors in wheat straw hydrolysate between the two evolved strains.

Furfural residue is the byproduct of furfural production from corncob, containing abundant cellulose, lignin and organic acids (Sun et al., 2019). The furfural residues provided by domestic enterprise contained 31.3 ± 1.4 mg/g of acetic acid and 5.5 ± 0.5 mg/g of HMF (Table 1). Some studies optimized the saccharification process of furfural residues (Lin et al., 2018; Zheng et al., 2015), but few studies tried to bioconversion of furfural residues to bioproducts, largely owing to the concentrated inhibitors contained therein. Due to the limited treatment methods, the piling, burying and burning of furfural residues seriously destroyed the ecological balances (Sun et al., 2019). The hydrolysate using 25 % (w/w) solids loading of furfural residues contained considerable high concentrations of acetic acid (13.9 ± 0.5 g/L), HMF (2.4 ± 0.1 g/L), and formic acid (2.5 ± 0.1 g/L) (Fig. 4**e-g**). We attempted to biodetoxify the furfural residues hydrolysate by *P. variotii*. The results showed that all of acetic acid, HMF and formic acid were degraded within 34 h by the evolved strains, at which time only 65.0 % of acetic acid was degraded by the parental strain. Similar to the biodetoxification in synthetic liquid medium, the evolved strain ZW70 exhibited a relatively better inhibitor degradation of acetic acid and furfural, outperforming the parental strain and evolved strain AC70.

Considering the differences in inhibitors degradation capacity between the evolved strains and parental strain, the transcriptional expressions of the key genes on the initial assimilation pathways of acetic acid, furfural, and HMF were analyzed using the quantitative real-time PCR (qRT-PCR) as shown in Fig. 5. The predicted initial metabolic pathways of acetic acid, furfural and HMF in *P. variotii* was conducted based on previous studies (Crigler et al., 2019; Koopman et al., 2010; Yi et al., 2019), which are relatively conserved (**Figure S2**) (see supplementary materials). It is worth noting that the qPCR between the evolved strain ad parental strain was conducted in the medium without containing the inhibitors according to the extensive literature summary (Liu et al., 2021; Lu et al., 2022; Sha et al., 2023; Wang et al., 2021; Zhou et al., 2021). The main reason to assay the transcriptional change in the inhibitors-free medium is to test the genetic stability of the evolved strains in terms of metabolism and gene expressions.

HMF and furfural are firstly converted to HMF alcohol and furfuryl alcohol by aldo–keto reductase/aldehyde reductase (*AKR/ARI*) and

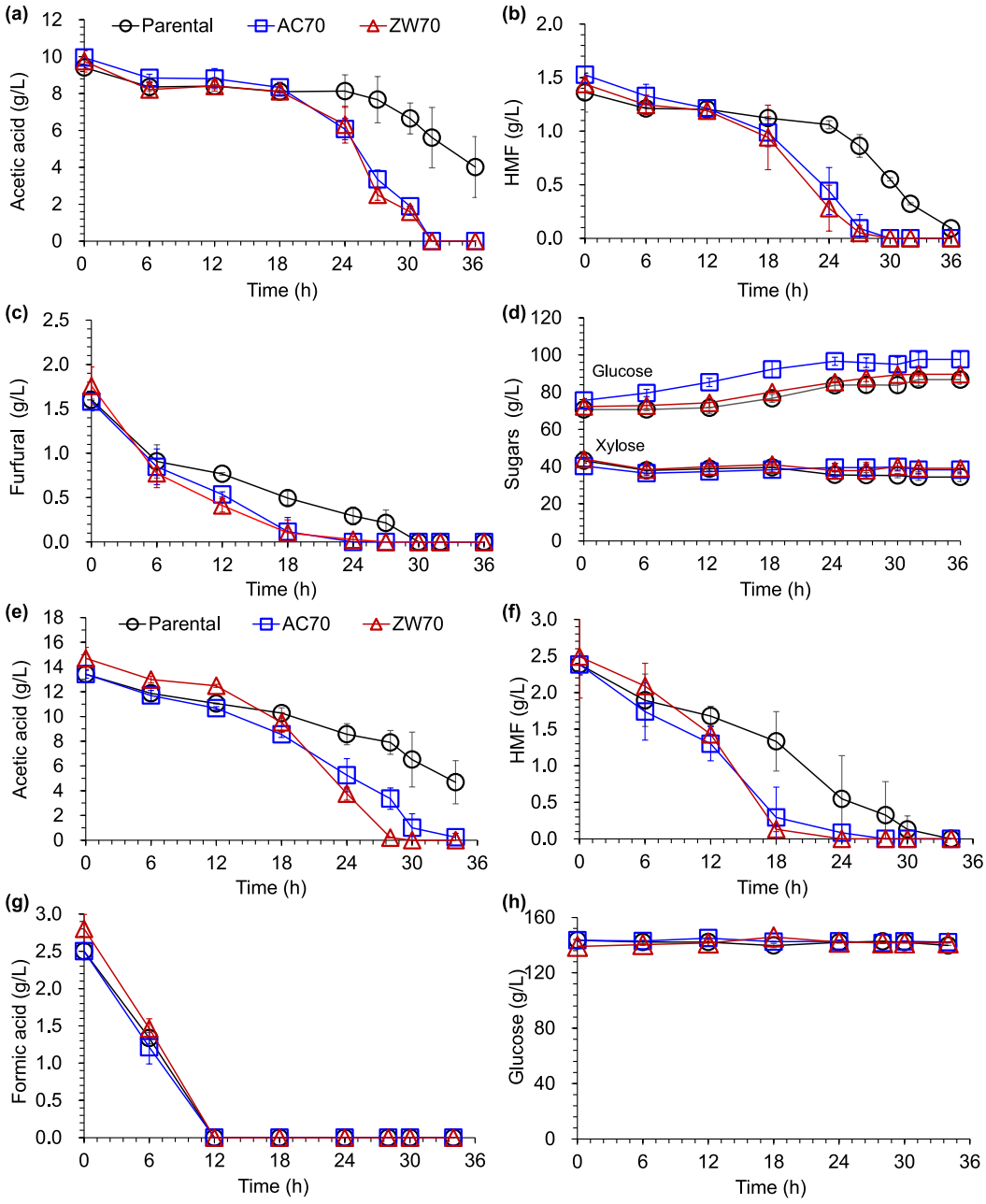

Fig. 4. Evaluations of biodetoxification performances of the parental and evolved strains in wheat straw hydrolysate (a-d) and furfural residues hydrolysate (e-f). Acetic acid conversion (a & e); HMF conversion (b & f); furfural conversion (c & g); sugars consumption (d & h). The cell growth was not detected due to the presence of solid particles. Each experiment was performed in triplicate. The error bar represents the standard deviation.

Table 1
The main compositions of pretreated wheat straw and furfural residues.

	Pretreated wheat straw[a]	Furfural residues
Cellulose (mg/g)	310.7 ± 5.2	474.7 ± 3.5
Glucose (mg/g)	30.8 ± 0.6	/[b]
Xylan (mg/g)	4.4 ± 1.3	/[b]
Xylose (mg/g)	136.7 ± 2.0	/[b]
Acetic acid (mg/g)	30.5 ± 0.2	31.3 ± 1.4
HMF (mg/g)	3.6 ± 0.1	5.5 ± 0.5
Furfural (mg/g)	8.9 ± 0.1	/[b]

[a] The raw wheat straw was pretreated on pilot scale. The conditions for dry acid pretreatment were 66.7 % (w/w) solids loading, 175°C, 5 min. The contents were based on dry weight.

[b] Where "/" indicates the contents below 1 mg/g.

alcohol dehydrogenase (*ADH*). The expression levels of *AKR* were significantly upregulated in both the evolved strain AC70 and ZW70 by more than 4 folds, while *ARI* was downregulated (Fig. 5**a**). The expression levels of three different *ADH* genes including alcohol dehydrogenase (*ADH*), alcohol dehydrogenase class V (*ADH-V*), and NADP-dependent alcohol dehydrogenase (*ADH8*) showed a relatively significant upregulation in *P. variotii* ZW70 and less significant regulation in *P. variotii* AC70, indicating the conversions of HMF and furfural into less toxic HMF alcohol and furfuryl alcohol was relatively active by the adaptive evolution in submerged liquid medium. Previous studies revealed that the over-expressions of the aldo–keto reductase gene in *Clostridium tyrobutyricum* (Suo et al., 2019) or NADPH-dependent alcohol dehydrogenase in *Zymomonas mobilis* (Wang et al., 2017) enhanced the reduction in the toxicities of furan aldehydes. The up-regulated expression levels of *AKR* and *ADH* improved the tolerance of

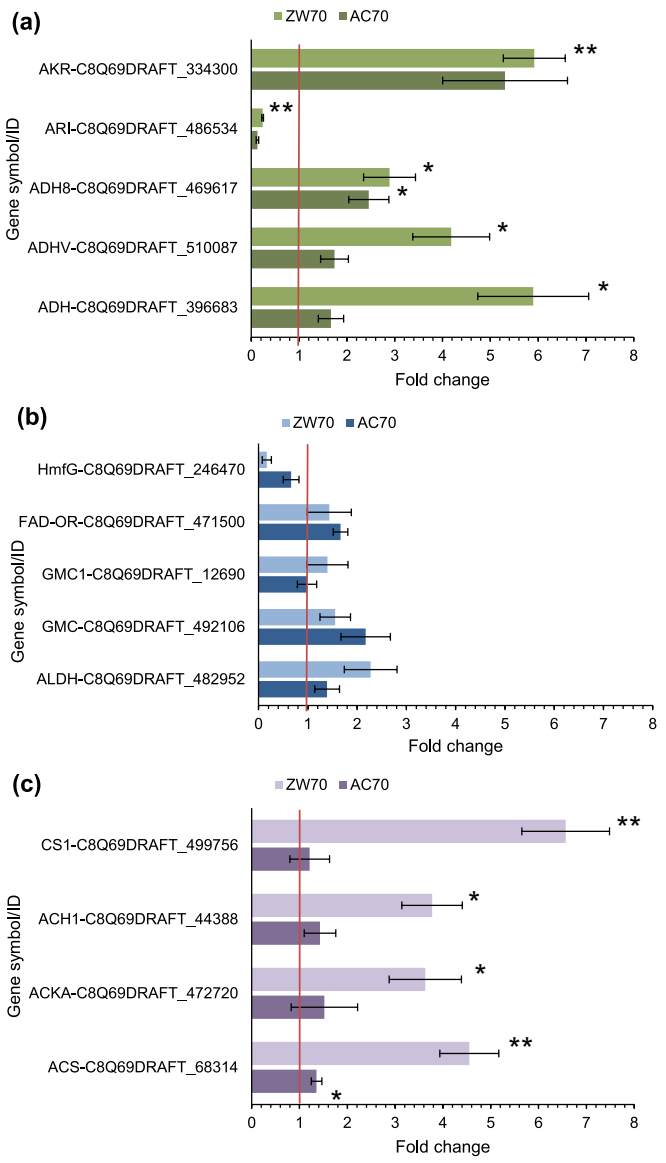

Fig. 5. The changes in the expression levels of the key genes related to the metabolic pathways of acetic acid, HMF and furfural in evolved strains compared to parental strain. (a) The changes in the expression levels of the key genes involving in furan aldehydes metabolism into alcohol form. (b) The changes in the expression levels of the key genes involving in furan aldehydes metabolism into furoic acid. (c) The changes in the expression levels of the key genes involving in acetate metabolism. Error bars represent standard deviation of the average of three biological replicates.

P. variotii to furan aldehydes inhibitors.

The conversions of furfural and HMF alcohols to furoic acid and HMF acid could be directly oxidized by glucose-methanol-choline oxidoreductase (*GMC* and *GMC1*) and aldehyde dehydrogenases (*ALDH*) (Fig. 5**b**). All the target genes on the two pathways were not significantly changed in expression levels in both the evolved strains, indicating the oxidations to acids were the slow conversion comparing with the reductions to alcohols. For HMF acid, two more steps are involved for the conversion to furoic acid by oxidation into 2,5-furan-dicarboxylic acid (FDCA) by *FAD-OR* gene encoding FAD-dependent oxidoreductase, then, the FDCA conversion to furoic acid is catalyzed by gene *HmfG* encoding the 3-octaprenyl-4-hydroxybenzoate carboxy-lyase-domain-containing protein. *FDA-OR* showed no upregulation, and *HmfG* showed down-regulation, indicating this conversion was a slow and depressed step. After the formation of furoic acid from furfural and HMF, furoic acid is assimilated to furoyl-CoA by acyl-CoA synthetase. Furoyl-CoA was converted sequentially to 5-hydroxy-2-furoyl-CoA, 2-oxoglutarate-CoA, 2-oxoglutaric acid, 2-oxoglutaric acid by acyl-CoA synthetase, furoyl-CoA synthetase, spontaneous reaction or nonspecific hydrolase, 2-oxoglutarate-CoA hydrolase, finally entered the central metabolism of TCA cycle for ultimate degradation. The ultimate degradation of furan aldehydes in *P. variotii* shared a similar metabolism pathway to that reported in *Pseudomonas putida* ALS1267 (Crigler et al., 2019). The previous studies showed that the activities of *ALDH* and *GMC* were severely inhibited by furan aldehydes, but *ADH* was more resistant to these inhibitors (Jilani et al., 2023). This may be the reason why the expression levels of the genes related to the oxidations of furans to acids were lower than the those of the genes related to the reductions to alcohols in *P. variotii*.

Acetic acid assimilation pathways include (i) the formation of acetyl-CoA by acetyl-CoA synthetase (*acs*) in cytoplasm with acetyl-AMP as an intermediate; (ii) the formation of acetyl-CoA by acetyl-CoA hydrolase (*ach1*) in mitochondria; and (iii) the formation of acetyl phosphate by acetate kinase (*ackA*) (Fig. 5**c**). The transcriptional levels of *acs*, *ach1*, and *ackA* were significantly upregulated by more than 3-fold in *P. variotii* ZW70 compared to parental strain. In contrast, insignificant upregulation was observed in *P. variotii* AC70. Acetyl-CoA is further utilized through condensation with oxaloacetate to citrate in mitochondria and enter the TCA cycle by the citrate synthase *CS1*. The transcriptional level of the gene *cit*1 encoding *CS1* indicated that *CS1* was significantly upregulated by more than a 5-fold increase in *P. variotii* ZW70. While insignificant upregulation was observed in *P. variotii* AC70. The up-regulations of *acs*, *ach1* and *cit1* enhanced the conversion of acetic acid to acetyl-CoA and final entrance to the central metabolic pathway in *P. variotii* ZW70. In summary, the evolutionary adaptation in liquid medium with inhibitors could better boost the acetic acid assimilation compared to that on solid substrate.

3.3. Fermentability of the biodetoxified hydrolysate for L-lactic acid production

The biodetoxified wheat straw hydrolysate and furfural residues hydrolysate were subjected to cellulosic lactic acid fermentation (Fig. 6**)**. The lactic acid titers reached 107.9 ± 0.4 g/L and 106.0 ± 1.0 g/L using the wheat straw hydrolysate detoxified by *P. variotii* AC70 and ZW70, which were 23.9 % and 27.1 % higher than that using the hydrolysate detoxified by parental strain (Fig. 6**a & 6c)**. The biodetoxification of wheat straw hydrolysate by evolved strains also improved the consumption of fermentable sugars of *P. acidilactici* ZY271. The complete glucose and xylose consumption were achieved within 48 h, approximately 12 h shorter than that using the hydrolysate detoxified by parental strain.

For the cellulosic lactic acid fermentation using the biodetoxified furfural residues hydrolysate (Fig. 6**b & 6d)**, the lactic acid titers reached 108.1 ± 0.1 g/L and 112.2 ± 0.3 g/L when the hydrolysate was detoxified by *P. variotii* AC70 and ZW70, which were 17.6 % and 22.0 % higher than detoxified by the parental strain. The residual acetic acid (~4 g/L) in the hydrolysate after the biodetoxification by the parental strain led to the reduced lactic acid fermentation efficiency. Additionally, the lactic acid titers using the hydrolysate detoxified by *P. variotii* ZW70 were slightly higher than detoxified by *P. variotii* AC70.

All in all, as the lignocellulosic biorefinery process towards industrial demonstration, the transportation of feedstock, pretreatment, saccharification, fermentation should be carried out as much as possible at higher solids loading. The high solids loading in biorefining process reduces water input, energy consumption, toxic wastewater emission, solid/liquid separation costs, carbon dioxide emissions, and improves the fermentation titers (Zhang et al., 2023a). However, on the other hand, the increase in solids loading leads to the decrease in wastewater discharge, much inhibitors generated in harsh pretreatment are shifted from the wastewater to the hydrolysate. The development of effective

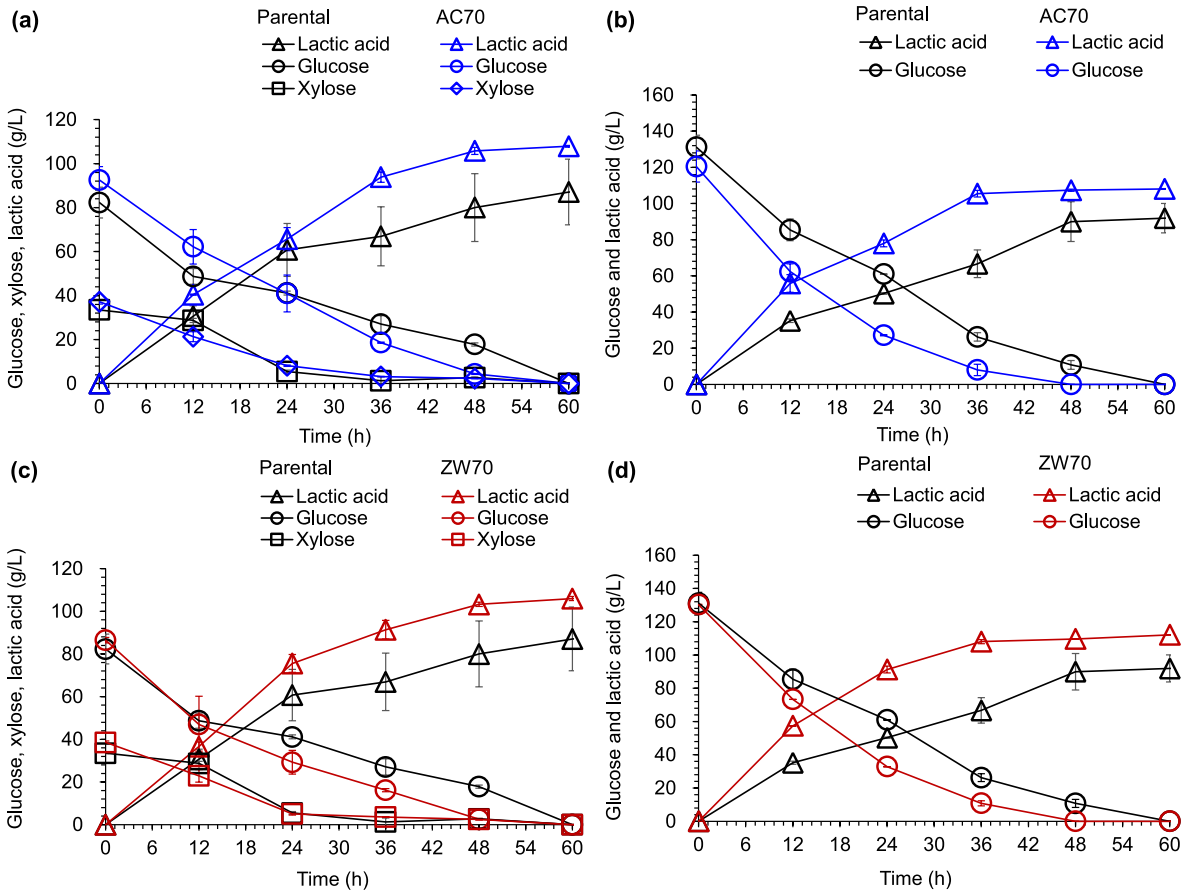

Fig. 6. Lactic acid fermentation performance using biodetoxified lignocellulosic hydrolysate. The lactic acid fermentation using wheat straw hydrolysate biodetoxified by *P. variotii* AC70 (a) and ZW70 (c) compared with parental strain. The lactic acid fermentation using furfural residues hydrolysate biodetoxified by *P. variotii* AC70 (b) and ZW70 (d) compared with parental strain. The fermentation was carried out in 3 L bioreactor at 42 °C with 300 rpm agitation by *P. acidilactici* ZY271. The fermentation pH was controlled at 5.5 by automatically adding 25 % (w/w) calcium hydroxide slurry. All data were repeated twice. Each experiment was performed in triplicate. The error bar represents the standard deviation.

detoxification methods has therefore become more prominent to facilitate the bioconversion of the lignocellulosic hydrolysate containing high titers of inhibitors cocktail in this scenario. This study provided a robust biodetoxification strain by long-term adaptive evolution. The evolved strain can degrade high concentration lignocellulose-derived inhibitors cocktail prior to fermentable sugars, which preserved the fermentable sugars during the biodetoxification. The high concentration inhibitors cocktail in the hydrolysates of wheat straw pretreated on pilot scale and furfural residues can be effectively removed by the evolved strain, successfully achieving the valorization of agro-industrial waste feedstocks. Furthermore, the verification of the genes identified with significant transcriptional changes is very helpful to understand how the adaptive evolution improved the inhibitor tolerance of *P. variotii*. However, the biodetoxification behavior is a very complicated phenomenon and the combined outcome of a metabolic network. The whole genome of *P. variotii* FN89 has not been sequenced and the genetic manipulation method had not been well established. Therefore, the verification may need a systematic work in the future when the genetic manipulation tools are ready. This study provided the initial information on the target genes, which are important for efficient biodegradation of high levels of inhibitors for the future elucidation of the mechanisms.

4. Conclusions

This study demonstrated the adaptive evolution in solid substrate and liquid medium could boost the biodetoxification capacity of *P. variotii*. Evaluations of biodetoxification revealed enhanced inhibitor conversion rates and cell growth in both evolved strains. Transcriptional analysis revealed the upregulation of the key genes involved in initial acetic acid assimilation and furfural/HMF conversion pathways, particularly in strain ZW70 obtained by evolution in liquid medium. Significant improvement in cellulosic lactic acid fermentation was highlighted using wheat straw and furfural residues hydrolysates detoxified by evolved strains compared to the parental strain. The study provided robust biodetoxification strains for bioconversion of lignocellulosic feedstocks.

CRediT authorship contribution statement

Agustian Chen: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Bin Zhang:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition. **Jie Bao:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.131351.

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