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Study on biodetoxification performance enhancement of *Paecilomyces variotii* in high inhibitors containing lignocellulose hydrolysates

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2024 年 6 月 3 日

Study on biodetoxification performance enhancement of *Paecilomyces variotii* in high inhibitors containing lignocellulose hydrolysates

Abstract

Utilization of lignocellulose biomass for producing value-added chemicals is favorable due to its sustainability, cost-effectiveness and non-competitiveness with food crops. Pretreatment, a prerequisite in the biorefinery processing chain to break the rigid structure of lignocellulose, generates high titers of inhibitory compounds such as acetic acid, 5-hydroxymethylfurfural (HMF) and furfural. These inhibitors negatively affect cell growth and fermentation efficiency of microbial fermentative strains. To overcome this barrier, a detoxification step is crucially important to remove all the inhibitors from pretreated feedstocks. Biodetoxification is a unique approach by utilizing specific microorganisms such as *Paecilomyces variotii* to biodegrade ultimately and quickly the inhibitors. However, general biodetoxification microorganisms have limited tolerance of very high concentrations of inhibitors in lignocellulose hydrolysates, and thus resulting in a decreased biodetoxification performance. This thesis focused on the development of robust strains to enhance the biodetoxification performance of current detoxification strains.

The first part of this thesis used the adaptive evolution strategy on *P. variotii* FN89 by gradually increasing inhibitor concentrations to boost its tolerance and degradation ability. Two stable strains, *P. variotii* AC70 and *P. variotii* ZW70, were obtained from the adaptive evolution separately using the solid and liquid medium. Compared with the parental strain, the evolved strains demonstrated up to 55% and 22% increase for the degradation of HMF and furfural, respectively in high inhibitors containing wheat straw hydrolysate, and more than 50% increase for acetic acid. The biodetoxified hydrolysates were used for L-lactic acid fermentation using the engineered lactic acid bacterium *Pediococcus acidilactici* ZY271, resulting in a 22% increase in lactic acid generation. Transcriptional analysis unveiled a significant upregulation of the genes associated with the initial conversion of acetate and furan aldehydes metabolism pathways by more than 3-*fold* in the evolved strain ZW70.

The second part of this thesis focused on the isolation of biodetoxification strains from corncob residues after used for furfural production, which contained high titers of acetic acid and HMF. A new biodetoxification strain *P. variotii* CC004 was isolated with a 40.39%

enhancement of HMF degradation and 25.64% enhancement of acetic acid degradation compared with the parental strain *P. variotii* FN89. The biodetoxified corncob residue hydrolysate was then used for L-lactic acid fermentation by *P. acidilactici* ZY271, resulting in a 12.18% increase in lactic acid accumulation than that of the *P. variotii* FN89. The transcriptional analysis of the isolate *P. variotii* CC004 showed upregulation-related genes of initial conversion of acetic acid and furan aldehydes metabolisms by more than 3-*fold*.

This thesis presents the candidate genes and robust strains of the outstanding inhibitor tolerance and biodetoxification performance in high inhibitors containing hydrolysates for efficient L-lactic acid biorefinery using lignocellulose feedstocks. By enhancing the detoxification performance of the existing detoxifying fungus *P. variotii* FN89 and evaluating the detoxification ability of newly isolated strains CC004, this thesis offers microbial catalysts for the refinement of biofuels and biobased chemicals.

Keywords: *Paecilomyces variotii*; lignocellulose; biorefinery; biodetoxification; adaptive laboratory evolution; L-lactic acid

强化宛氏拟青霉在高抑制物浓度纤维素水解液的生物脱毒性能研究

摘要

利用木质纤维素生物质生产高附加值的生物基化学品具有可持续性、低成本高效益和不与粮食类物料竞争的优势。预处理是生物炼制过程的先决条件，用于破坏木质纤维素的致密结构，但该过程会产生高浓度的乙酸、5-羟甲基糠醛和糠醛等抑制化合物。这些抑制物会对发酵菌株的细胞生长和发酵产生负面影响。为了克服这一障碍，需要去除预处理后所产生的抑制物，所以脱毒步骤至关重要。生物脱毒是一种利用特定微生物（如*Paecilomyces variotii*）降解抑制物的方法。然而，现有的生物脱毒菌株对高浓度抑制物的耐受性有限，其在高浓度抑制物的条件下脱毒能力下降。本论文致力于开发耐受高浓度抑制物的菌株，同时增强现有脱毒菌株的脱毒性能。

该论文的第一部分对 *P. variotii* FN89 进行适应性进化，以提高其抑制物耐受性和降解能力。在固体培养基和液体培养基进行适应性进化过程中，本研究获得两株稳定脱毒菌株 *P. variotii* AC70 和 *P. variotii* ZW70。与出发菌株相比，在含有高抑制物的小麦秸秆糖化液中，进化菌株降解 HMF 和糠醛的能力都提高了 55%和 22%，降解乙酸的能力提高了 50%。利用生物脱毒后的糖化液，乳酸片球菌 *Pediococcus acidilactici* ZY271 乳酸产量增加了 22%。转录分析揭示了与乙酸和呋喃醛代谢途径相关的基因在进化菌株 ZW70 中的上调三倍以上。

论文的第二部分致力于从用于糠醛生产的、含有高浓度的乙酸和 HMF 玉米芯渣中筛选脱毒菌株。本研究筛选到一株脱毒菌株 *P. variotii* CC004，其 HMF 和乙酸降解速率分别比出发菌株 *P. variotii* FN89 高出 40.39%和 25.64%。利用生物脱毒后的玉米芯渣糖化液进行乳酸发酵，*P. acidilactici* ZY271 的乳酸产量增加了 12.18%。对新筛选的菌株 *P. variotii* CC004 进行转录分析，结果表明与乙酸和呋喃醛代谢转化相关的基因有所上调三倍以上。

本论文通过适应性实验进化手段强化了现有生物脱毒菌株的抑制物转化能力。通过对现有脱毒真菌 *P. variotii* FN89 生物脱毒能力强化和对新分离菌株的生物脱毒能力测试，为生物燃料和生物基化学品的炼制提供新的菌株催化剂。

关键词: *Paecilomyces variotii*；木质纤维素；生物炼制；生物脱毒；适应性进化；L-乳酸

Table of Contents

Chapter 1 General Introduction

1.1 Lignocellulose biomass

The adverse environmental effects caused by the current continued petrochemical materials utilization emphasize the need for alternative sustainable materials to address climate change and ensure energy security. The demand for green materials is a critical challenge with implications for global stability, economic prosperity, and quality of life. In this term, the development and utilization of plant-based feedstocks and biomass processing in biorefineries, particularly for producing value-added bioproducts, such as biofuels and chemicals, are considered a viable approach and viewed as means to diversify energy supply, decrease reliance on fossil fuels, and minimize environmental harm. Lignocellulose, as a product of the photosynthesis process, is identified as a plentiful biomass that can be utilized as a promising resource to achieve the goal of a sustainable global carbon economy[1, 2].

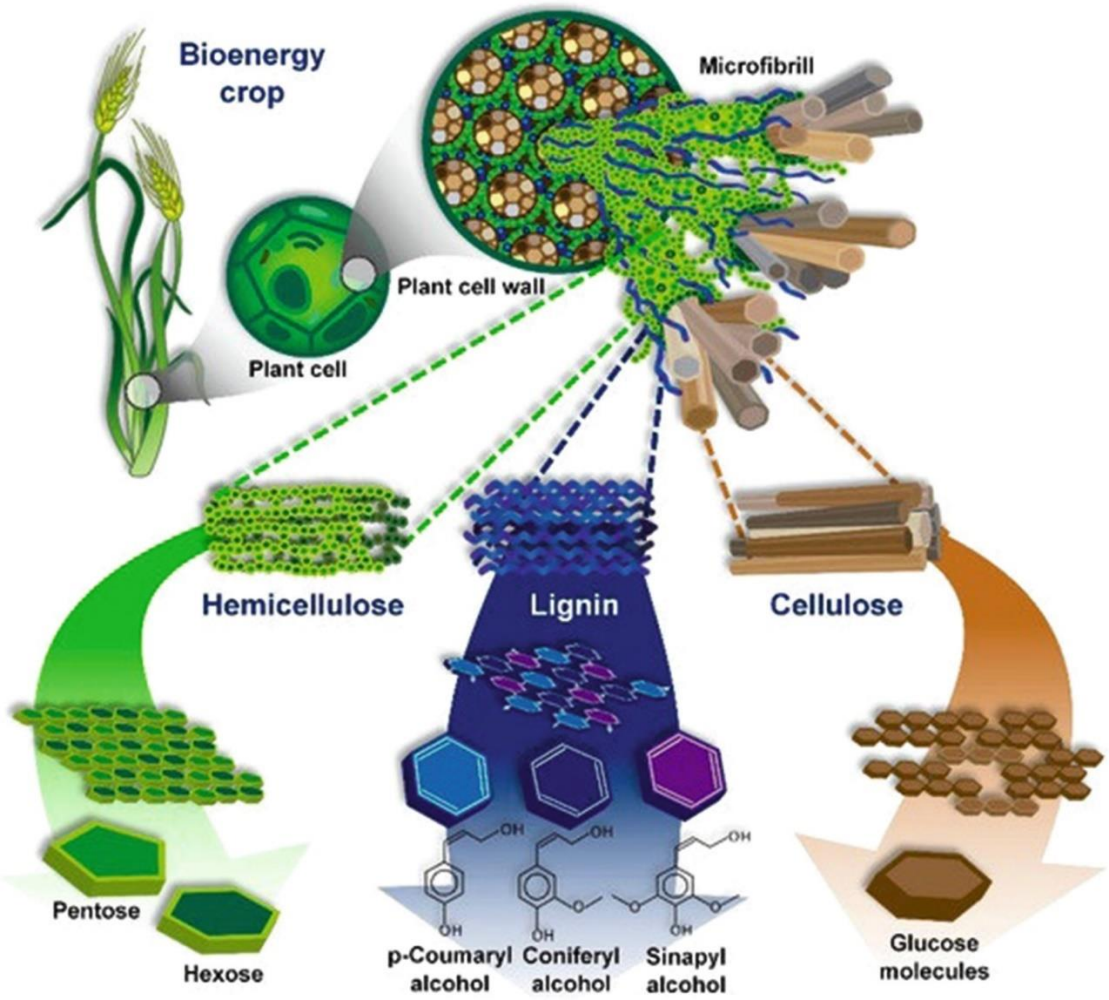


Figure 1 Structure of lignocellulosic biomass[3]

Lignocellulosic biomass is a component of agricultural waste, including forestry, fruits, grain, and vegetables. Agricultural wastes such as corncob, corn and cereal straw, sugar beet parts, potato haulms, rapeseed and sunflowers oil pressing residue, paper industry residues, sawmills and paper waste are the main supplies of lignocellulosic materials. Commonly, lignocellulosic biomass consists of three main biopolymers: cellulose, hemicellulose and lignin[4]. **Figure 1** presents the overall matrix structure of lignocellulosic biomass. Cellulose, with stable glucose chains linked by β-glycosidic bonds, is the most resistant structure and recalcitrant against external chemical and biological attacks. Hemicellulose, featuring branched chains of sugars, has lower molecular weight and degrades more easily than cellulose, consists of different sugar monomers, and includes xylose, mannose, galactose, rhamnose and arabinose. Lignin, rich in polyphenolic constituents, forms a dense matrix linking with hemicellulose, providing structural integrity to plants. Cellulose and hemicellulose can be hydrolyzed to the fermentable sugars for microbial fermentation. While lignin resists efficient biodegradation and interferes with hydrolytic enzymes, increasing the cost of enzymatic hydrolysis and bioproduct production[5-10].

Different type of lignocellulosic biomass consists of different biochemical compositions, and their content depends on the nature of the feedstock. In general, cellulose dominates 30–70% of the biomass component, while hemicellulose and lignin share 15–30% and 10–25% of lignocellulosic biomass, respectively, as presented in Table 1[11].

Table 1 Major compositions of various lignocellulosic biomass[10]

Feedstocks		Cellulose	Hemicellulose	Lignin
Agricultural residues	Wheat straw	39.6	26.6	21
	Wheat bran	42.5	21.2	3.4
	Corn stover	36.8	30.6	23.1
	Rice straw	32	18	11.2
	Barley straw	37.5	25.3	16
	Maize stover	41.7	18.9	26.1
	Maize bran	39.8	29.7	2.6
	Rye straw	38	36.9	17.6
Hardwoods	Poplar	44.5	22.5	19.5
	Eucalyptus	54.1	18.4	21.5
Softwoods	Spruce	45.5	22.9	27.9
	Pine	43.3	21.5	28.3

1.2 Pretreatment

In chemical production, the main processes encompass biomass handling, size reduction, pretreatment, detoxification, hydrolysis and fermentation. Nevertheless, variations may occur in the sequence and nature of these processes and based on factors, such as the nature of the biomass source, the chosen conversion route to bioproducts, and the specific type of bioproducts, which can be adapted and customized to achieve better optimization[12].

In biomass handling, a mechanical step is crucial to enhance bulk density and flowability for efficient transport. Typically, 3–8 mm particles by grinding are conducive to the formation of higher density pellet or briquette. Proper biomass storage is crucial to maintain continuous biochemical production in the face of unpredictable feedstock availability. The biomass storage commonly used is outdoor wood chunk storage, but sunlight exposure can induce terpene emissions. Specialized silos and facilities protect biomass from weather, rodents, and microbial growth, preventing substrate loss and self-ignition. Maintaining dry conditions minimizes microbial activity during storage[13-15].

Pretreatment is a crucial step of lignocellulose bioconversion, which facilitates disruption of the rigid structure of the feedstock to increase enzyme accessibility and reduce lignin's shielding effect for efficient conversion in the hydrolysis step[16, 17]. Ideal pretreatment possesses several target features for efficiency, including maximizing sugar yields, facilitating high digestibility of cellulose during subsequent enzymatic hydrolysis, minimizing inhibitory compound formation, resulting in low moisture content or a high solids concentration and a substantial concentration of liberated sugars in the liquid fraction, demanding low energy input or allow for energy re-use in other process steps as secondary heat, requiring low capital and operational costs to ensure economic feasibility and less wastewater[18-21].

The main pretreatment classes for lignocellulose biomass include mechanical, chemical, physiochemical and biological. Mechanical methods, including ball, hammer, roller, colloid, vibrio energy, chipping, milling and grinding, aim to reduce particle size and crystallinity, enhance hydrolysis yield, and reduce digestion time to increase the surface area of cellulose, and facilitate enzymatic hydrolysis. However, there are limits to effectiveness, and power requirements can limit economic feasibility[20-27].

Chemical pretreatment methods include dilute-acid, organosolv, ionic liquids, alkaline and lime pretreatment. Alkaline pretreatment involves soaking in alkaline solutions like sodium or potassium hydroxide to increase internal surface area and decrease polymerization and crystallinity. Breaking bonds between lignin and carbohydrate polymers is for making

carbohydrates more accessible to hydrolytic enzymes. This method is effective on certain raw materials, such as agricultural residues and herbaceous crops, due to their lower lignin content [28, 29]. Dilute acid pretreatment utilizes mineral acids like sulfuric acid to cleave constituents into smaller molecules, followed by heating within a specific temperature range between 140 and 200 °C. The acid indiscriminately acts on cellulose, hemicellulose, and lignin, resulting in various compounds, which are toxic substances to fermenting microorganisms[30-32]. The organosolv process utilizes organic or aqueous-organic solvent mixtures such as methanol, ethanol, acetone, etc., with added mineral acid to break internal lignin and hemicellulose bonds. Recovering hydrolyzed lignin in the organophilic phase is emphasized, with thorough washing required to remove inhibitors before enzymatic hydrolysis and fermentation. The flammability, explosiveness and recovery of the solvent are essential factors for economic and environmental reasons[33]. Dilute acid pretreatment (DAP) is an effective method for biomass processing, but it has drawbacks such as high wastewater generation, inhibitor compound production, and reactor corrosion. To address these issues, a modified version called dry dilute acid pretreatment (DDAP) was developed. DDAP reduces freshwater and hot water steam usage during pretreatment, resulting in a solid/liquid ratio of 1:0.5, compared to the regular ratio of 1:10. This modification eliminates free acid solution during pretreatment by absorbing all water streams into the lignocellulose solids. Consequently, wastewater generation is significantly reduced. Additionally, inhibitor generation is mitigated by introducing biodetoxification by *Amorphotheca resinae* ZN1 fungus on the pretreated solids, without requiring freshwater input or generating wastewater[236].

Steam pretreatment, hydrothermolysis, wet oxidation and ammonia-based methods, such as ammonia fiber explosion (AFEX) and ammonia recycle percolation (ARP), belongs to physiochemical pretreatment. Steam pretreatment involves a catalyst (H_2SO_4 or SO_2) and utilizes high-pressure saturated steam at high temperatures[34, 35]. Hydrothermolysis is similar to steam pretreatment but employs hot water with lower temperatures. This method yields solubilized oligomeric sugars and demands more energy in downstream processes[36, 37]. Wet-oxidation pretreatment, involving water and air or oxygen at temperatures of 120-200 °C, is suitable for feedstock with lower lignin content[38, 39]. AFEX involves liquid ammonia under moderate temperature and elevated pressure. This method yields materials with increased water-holding capacity and digestibility and is suitable for agricultural waste due to its lower lignin content [40-43]. ARP, an ammonia recycling percolation method at elevated temperatures, efficiently delignifies hardwood and agricultural residues[44, 45]. These pretreatment methods vary in effectiveness depending on the feedstock composition and desired properties of the

resulting materials, with some methods more suitable for specific types of biomass due to their lignin content and structural characteristics.

Biological pretreatment involves the application of lignin-degrading microorganisms, such as white- and soft-rot fungi, to lignocellulose materials or the utilization of industrial enzymes like cellulase, xylanase and lignolytic enzymes such as laccase, lignin, and manganese peroxidase to disrupt lignocellulosic barrier, especially lignin for enzymatic cellulose hydrolysis[46, 47]. White-rot fungi are particularly effective in this process and produce enzymes like peroxidases for lignin degradation[48]. Laccases require redox mediators, while manganese peroxidase and versatile peroxidase use Mn^{3+} ions and aromatic radicals [49, 50]. Environmentally friendly, energy-saving, and chemical-free biological pretreatment has slow process rates and material loss as microorganisms consume hemicellulose, cellulose or lignin. The method necessitates the combinations with other pretreatment methods[51]. Though the biological treatment rate is slower than other methods, the interest has grown in exploring alternative microorganisms like brown rot fungi for enhancing enzymatic hydrolysis.

The demand of optimizing a desired goal, such as achieving high degrees of solubilization of hemicelluloses and lignin, has additional effects. In this case, it leads to the degradation of the solubilized fragments due to the harsh conditions they undergo. The quantity and type of degradation products formed, some of which hinder downstream biocatalytic processes. The degradation product formations depend on the pretreatment method and conditions, namely the generation of toxic compounds that are hindrances to the enzymes or the microorganisms used for the subsequent fermentation process. These inhibitors need to be degraded before the fermentation process to obtain optimal productivity in fermentation.

1.3 Diversity of lignocellulose-derived inhibitors

Various pretreatment methods aim at enhancing sugar recovery and improving subsequent chemical production from cellulosic materials. Despite advancements, challenges persist in enzymatic hydrolysis and fermentation processes. Pretreatment processes remove hemicellulose and partially solubilize lignin to increase enzyme accessibility to cellulose, thereby enhancing conversion yield. However, undesirable lignocellulose-derived compounds, such as furan aldehydes, organic acids, phenolic compounds, and soluble sugars, can be released during pretreatment[49, 50, 52-56]. The main components of hydrolysis products of lignocellulose biomass are glucose, galactose, mannose, xylose, arabinose, phenols and acetic acid (Figure 2). Harsh pretreatment conditions lead to the generation of inhibitors that hinder fermentable sugar conversion of hydrolytic enzymes. The classification of these inhibitors

includes aliphatic acids (such as acetic, formic and levulinic acids), furan aldehydes (5-hydroxymethylfurfural [HMF] and furfural), and phenolics[57]. Different lignocellulose biomass materials and pretreatment methods result in distinct formation of inhibitor compounds as described in Table 2.

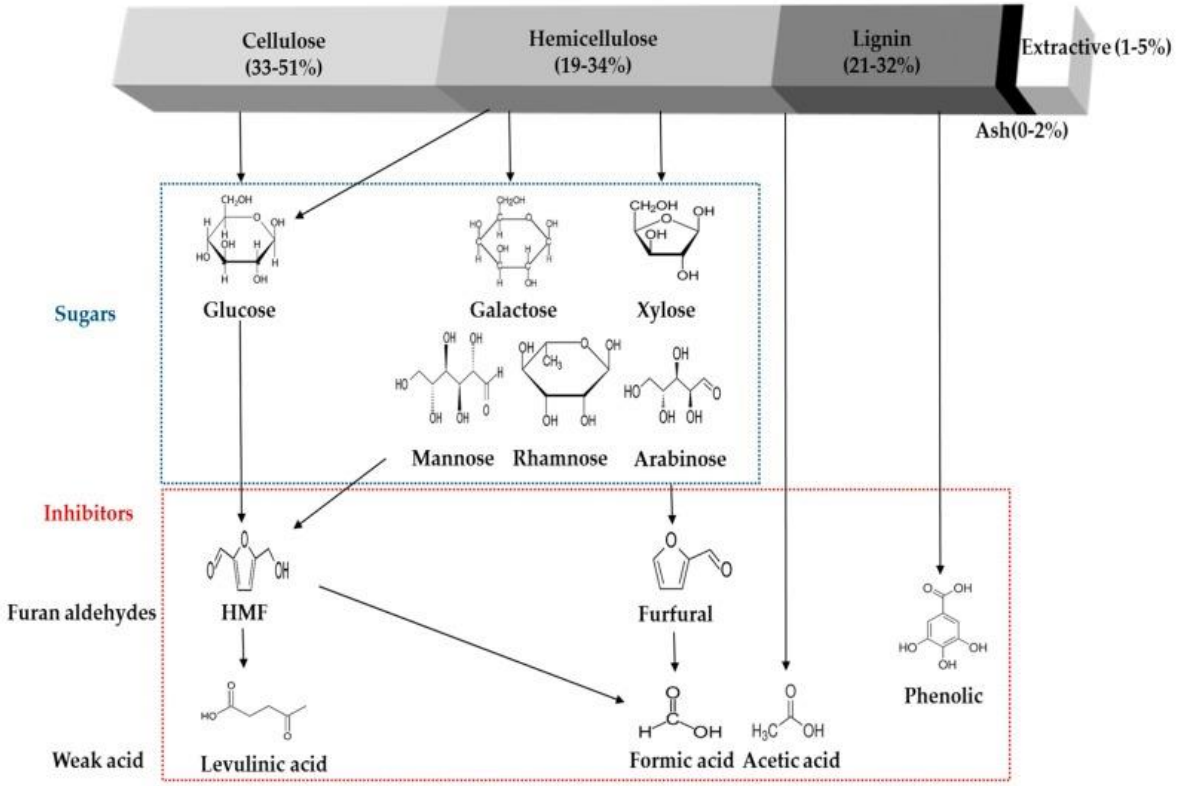


Figure 2 Brief scheme of major inhibitory compounds formation pathway from three major biopolymers[57].

1.3.1 Aliphatic acids

Lignocellulose hydrolysates contain primary short-chain aliphatic acids, including acetic acid, formic acid, and levulinic acid. These acids are generated from hydrolysis and acid-catalyzed thermochemical degradation of polysaccharides[58, 59]. Acetic acid primarily originates from hemicellulose hydrolysis, while formic acid and levulinic acid are products of 5-hydroxymethylfurfural (HMF) degradation. Formic acid is an acid-catalyzed thermochemical degradation product of furfural and HMF, while levulinic acid is a product generated by the degradation of HMF through rehydrating catalytically[60, 61]. The inhibition mechanism of weak acids is due to undissociated aliphatic acids diffusion through cell membranes via passive diffusion that may lead to cell death. Cells will rapidly generate ATP to maintain intracellular cells, leading to anaerobic respiration and ethanol production at the expense of biomass.

Dissociated acids face difficulty, although acetic acid can enter via facilitated diffusion using aquaglyceroporin Fps1p[62, 63]. In addition, dissociation forms of these small organic acids on cell membrane may lead to an influx into cytosols and cause disturbance in ion transportation, resulting in inhibition of cell growth, regeneration and metabolism[25, 64, 65]. Toxicity occurs when diffusion rates of aliphatic acids exceed efflux rates, causing a permanent pH drop and halting cellular processes. Cells will utilize active transport and ATPase mechanisms to expel dissociated acids and protons at the expense of ATP, and this will impact other energy-dependent processes like cell growth and fermentation. Other toxicity mechanisms of acids are also related to uncoupling and anion accumulation in the cells. Uncouplers are amphiphilic molecules that disrupt the proton gradient over the inner mitochondrial membrane and inhibit ATP regeneration. Some aromatic carboxylic acids, such as salicylic acid, may act as uncouplers[66-68]. Formic acid has a lower pKa value than acetic and levulinic acids, which possess higher toxicity [69]. The level of acid toxicity to microbial growth is related to its concentration and the acid chain length, in which the longer chain length is more hydrophobic, and the branched-chain is less toxic due to its decreased hydrophobicity[70]. The concentration of aliphatic acids varies depending on feedstock and pretreatment severity. Softwood hydrolysates typically have lower aliphatic acid content and are more beneficial for ethanol yield[69, 71].

Table 2 Overview of formation and source of inhibitory compounds generated from lignocellulose biomass by different pretreatment methods.

Pretreatment method	Feedstock	Inhibitors	Reference
Steam explosion	Olive tree pruning	Acetic acid, formic acid, furfural, HMF	[72]
Liquid hot water (LHW)	Sugarcane bagasse	Acetic acid, HMF, furfural, phenolics, gluco-oligomers, xylo-oligomers	[73]
Ionic liquids	Sugarcane bagasse	Acetic acid, HMF, furfural	[74]
Microwave-assisted-dilute ammonia pretreatment	Sorghum	Acetic acid, formic acid, furfural, HMF	[75]
Sulfuric acid pretreatment	Miscanthus, wheat straw	Acetic acid, HMF, furfural	[76]
Sodium hydroxide	Bana grass	Acetylaldehyde, phenolics	[77]
Ozone pretreatment	Wheat straw	Furfural, formic acid,	[78]

		acetic acid	
Milled and steamed treatment	Corn stover	Cello-oligosaccharides, xylo-oligosaccharides	[79]
Alkaline organosolv-pretreated	Poplar	Aliphatic acids, furan, phenol	[80]
AFEX			

1.3.2 Furan aldehydes

Furfural and HMF are common furan aldehydes derived from the degradation of pentose and hexose, respectively, during the pretreatment process as the result of acid-based and hydrothermal pretreatment processes of lignocellulose biomass[81-84]. Mechanisms toxicity of furan aldehydes are inhibiting glycolytic and fermentative enzymes in a dose-dependent manner, causing single-strand breaks of double-stranded DNA, reducing intracellular ATP and NAD(P)H levels, redirecting cell energy flux to damage repair, causing negative impacts on organelles and membranes, inducing intracellular reactive oxygen species (ROS) accumulation, damaging mitochondria, vacuole membranes, actin cytoskeleton, and nuclear chromatin and potentially damaging cell membrane through hydrophobicity[85-93]. Furthermore, the interaction of furan aldehydes with other compounds present in lignocellulose hydrolysate could cause synergistic effects and enhance the toxicity of other inhibitory compounds, such as acetic acid and phenols[94, 95]. These negative impacts will severely inhibit the cell growth of fermentative microbes and decrease the sugar uptake as the substrate of fermentations, resulting in low productivity of desired product formation[31]. Factors such as the nature of the lignocellulose biomass and the severity level of pretreatment methods affect the concentration of furan aldehydes in pretreated feedstock[96].

1.3.3 Phenolic compounds

Common phenolic compounds present in lignocellulose biomass consist of ferulic acid, vanillic acids, 4-hydroxybenzoic acid, guaiacol, catechol, vanillin, syringic aldehyde and 4-hydroxylbenzaldehyde which are formed during lignin degradation in pretreatment process[97, 98]. The toxicity mechanisms of phenolic compounds include their ability to penetrate cell membranes, increase membrane fluidity, promote integrity loss of membranes, damage cellular membranes that will lead to intracellular component leakage, and alter the ratios of protein-to-lipid[99-101]. Factors that affect the toxicity level of phenolic compounds are the hydrophobicity level and position of the substituents, with lower molecular weight compounds being more toxic due to rapid diffusion into cells and inhibiting glucose assimilation[97, 102-104]. Besides, phenolic

compounds could also elevate reactive oxygen species levels in cells, causing cytoskeleton damage, DNA mutagenesis, and programmed cell death[99]. Phenolic compounds could also cause electrochemical gradient alteration, especially weak acidic phenolics that can alter the electrochemical gradient by transporting protons back across mitochondrial membranes[105, 106]. The biomass species and solid loadings determine the concentration and the type of phenolic compounds[107].

1.4 Detoxification of inhibitors from pretreated lignocellulose biomass

The selection of lignocellulose feedstock and pretreatment conditions might affect the concentration and type of the inhibitors, such as opting for less recalcitrant feedstocks and mild pretreatment conditions[20]. Many progresses have been engaged to mitigate the negative impact of inhibitor hindrances, including chemical (neutralization, calcium hydroxide over-liming, activated charcoal treatment, ion exchange resins, and extraction with ethyl acetate), biological (enzymatic mediated using laccase, lignin peroxidase, in-situ detoxification, in-situ microbial detoxification, etc.) and physical (evaporation, membrane separations) methods. These methods vary in effectiveness and impact on hydrolysate chemistry and fermentability[31, 58, 108-110].

1.4.1 Physical methods

Evaporation method

Evaporation is carried out under vacuum conditions to effectively remove volatile inhibitors such as acetic acid, furfural and vanillin in the lignocellulosic hydrolysate. The method demonstrated a complete removal of furfural and HMF by 4% upon evaporation of 90 % of the initial volume. Evaporation is considered to be effective in removing volatile compounds like acetic acid, furfural, and vanillin but retains non-volatile toxic compounds[111].

Membrane separations method

This method employs adsorptive microporous membranes with surface functional groups attached to their internal pores, such as Sartobind Q, that are capable of removing cell wall-derived inhibitors from lignocellulose acid hydrolysates, offering improvements over traditional ion-exchange resins. The process involves pumping the feed through the membrane pores to bind solutes predominantly by convection. This results in a significant reduction in processing time. The adsorptive membranes exhibit a substantial drop in pressure for flow compared with typical packed beds. Additionally, membrane separation is preferred and selected to remove inhibitors from sulfuric acid-derived hemicellulosic hydrolysates, demonstrating effectiveness in removing various acids and furans[112-114]. Nanotechnology

applications highlight the separation and low energy consumption characteristics[115, 116].

Adsorbent method

The adsorbent method involves adding adsorbents like activated carbon into pretreatment solutions to precipitate inhibitors as a removal mechanism. Additionally, activated carbon is favorable due to its strong adsorption and low cost. New adsorbents like cross-linked polyethyleneimine (PEI) demonstrate promising removal capability. Selective adsorption of furan, fatty acids, and phenolic substances by adsorption resins and PEI is observed, with subsequent desorption for substance recovery. The method reduces the total mass of phenolic and furan aldehydes but may lead to the loss of higher reducing sugars[117-121].

Solvent extraction method

The solvent extraction method employs solvents, such as n-hexane, chloroform, and ethyl acetate, to separate inhibitors from fermentation broth based on their solubility. Ethyl acetate could effectively remove peracetic acid, furfural, vanillin, and 4-hydroxybenzoic acid in lignocellulose hydrolysate[31]. Different extractants have varying removal effectiveness on inhibitors, with n-hexane having poor removal capability for some inhibitors. While ethyl acetate and chloroform are effective for furfural but less so for phenolic compounds. Solubility differences in organic solvents result in varying extraction effects on different substances, with xylose minimally affected due to its insolubility in organic solvents[122].

1.4.2 Chemical methods

Neutralization

The neutralization is employed by neutralizing highly acidic hemicellulosic hydrolysates before fermentation using agents, preferably calcium hydroxide or sodium hydroxide, followed by a pH value adjustment range of around 6.0-7.0. During the process, only furfurals and phenolics can be removed from the pretreated feedstock by precipitation to some extent.

Calcium hydroxide over-liming

This method is performed by over-liming with high pH and temperature, and its applicability is a promising detoxification method for sulfuric acid-pretreated hydrolysates. This method is more effective in removing volatile inhibitory compounds such as furfural and HMF but causes a sugar loss (~10%). The byproduct formation is gypsum and converted from dried calcium hydroxide[123-127].

Activated charcoal treatment

This chemical detoxification method aims for cost-effective detoxification of hemicellulose hydrolysates with the mechanism of employing activated charcoal to remove

inhibitors such as furan and phenolic compounds. The characteristic of activated charcoal is its high capacity to absorb compounds without affecting sugar levels. This method depends on pH, contact time, temperature and the charcoal-to-liquid ratio[128-131].

Ion exchange resins

This method can remove lignin-derived inhibitors, acetic acid, and furfural with optimal pH adjusted to 10. Ion exchange resin encounters issues as cost-ineffective due to significant base chemical usage. This method could enhance fermentation productivity but cause a substantial loss of fermentable sugars. Due to its cost-effectiveness, this method has limited feasibility for commercial industrial purposes[132].

1.4.3 Biological methods

Biodetoxification methods offer better economic feasibility and environmental friendliness than physical and chemical detoxification treatments for detoxifying lignocellulose hydrolysates. The biodetoxification approach utilizes living microbes or microbial enzymes to modify the chemical nature of inhibitors in the hydrolysates during cultivation. However, these methods are slow rate and result in the loss of fermentable sugars that are considered unavoidable[58].

Microbial detoxification

In this approach, microorganisms, including yeast, fungi and bacteria, are employed to degrade inhibitors present in pretreated feedstock during cultivation before the fermentation process. These microorganisms can detoxify inhibitory compounds in nature. The method has advantages, including its specificity, and eco-friendliness, and potentially offers simultaneous detoxification and bioproduct production. In contrast, it has drawbacks in slow rate processing time. Notably, various studies have explored the efficacy of biological detoxification methods, showcasing promising results. As illustrations for thermophilic detoxification bacteria, detoxification of waste house wood (WHW) hydrolysate using the thermophilic bacterium *Ureibacillus thermosphaericus* has proven to be able to degrade furfural, HMF, and phenolic compounds. The bacteria exhibited rapid cell growth and consumed less than 5% of fermentable sugars[133]. In addition, fungal detoxifications have been reported in *Coniochaeta ligniaria* (NRRL30616) to metabolize furfural, HMF, aromatic, aliphatic acids, and aldehydes present in corn stover hydrolysate. High-pressure liquid chromatography-ultraviolet-tandem mass spectrometry (HPLC-UV-ms/MS) analysis demonstrated the removal of inhibitory side-products during fungal growth and enhanced subsequent ethanol fermentations[134]. *Trichoderma reesei*, a filamentous soft-rot fungus, was also reported to be capable of degrading

inhibitors in willow hemicellulose hydrolysate, resulting in improved ethanol productivity and yield. However, the growth rates of these fungi were relatively low, and they assimilated sugars from hydrolysates, impacting fermentable sugar availability[135]. For yeast detoxification, *Issatchenkia occidentalis* and *Iris orienalis* were identified as yeast isolates with the capability of detoxifying sugarcane bagasse hydrolysate, leading to increased xylitol productivity and yield in subsequent fermentations[136]. Furthermore, *Saccharomyces cerevisiae* mutants were also employed to remove acetic acid from hardwood-spent sulfite liquor, achieving successful detoxification and enhanced ethanol fermentation[137]. Anaerobic bacteria such as *Desulfovibrio furfuralis* could degrade furfural to acetate, CO_2 and methane and offer potential solutions for furfural detoxification in lignocellulosic biomass conversion processes[138, 139].

Enzymatic detoxification

Laccase and peroxidase from white rot fungi, such as Glac15 in *Ganoderma lucidum* 77002, were widely studied and employed for detoxification to remove 84% of phenolic compounds. They effectively remove phenolics from lignocellulose hydrolysates and improve ethanol productivity[140]. Enzymatic detoxification is considered a preferred and potentially cost-effective biotechnological route. Enzymatic detoxification offers advantages such as possible operation at higher temperatures than optimal temperatures for microorganism growth, rapid detoxification due to its high catalytic efficiencies, broad substrate specificity and low utility costs owing to mild conditions. However, drawbacks include prolonged incubation periods and high enzyme production costs despite efforts in biotechnology to reduce them[58]. Comparisons with other detoxification methods highlight laccase efficiency in removing inhibitors. However, the scalability and cost-effectiveness of laccase production remain challenges addressed through the genetic engineering of yeast hosts for large-scale enzyme production[141].

1.5 Enzymatic hydrolysis

Enzymatic hydrolysis of lignocellulosic biomass is a promising approach for sustainable cellulosic bioproduct production. Enzymatic hydrolysis, a heterogeneous catalytic process, involves molecular mass transfer and reaction kinetics. The process is complex due to the intricate lignin-cellulose-hemicellulose network and is affected by various factors[142]. Recent studies have focused on optimizing this process for enhanced conversion efficiency and cost reduction. Moreover, experimental methods have evolved from traditional approaches to high-throughput screening using microarray technology to optimize pretreatment parameters such as reagent concentration, substrate particle size, reaction temperature, and time[143-146]. Analyses of enzyme-substrate interactions optimize reactions, with theoretical models to elucidate mass

transfer mechanisms at the molecular level. Accessible cellulose surface area and pore volume emerge as critical parameters limiting hydrolysis. Mechanistic steps include enzyme diffusion into substrate particles, adsorption onto sites, and subsequent hydrolysis reactions. The diffusion rate is affected by factors, such as concentration gradient and temperature and determines the hydrolysis degree. Enzyme diffusion is a key rate-limiting step with a minor impact on particles smaller than 5×10^{-3} cm. The larger particle size and pre-hydrolysis stages are affected by diffusion and impact the material selection and prediction of the reaction[142].

Cellulose, a linear polymer of β-d-glucose molecules, is insoluble in water due to anhydrous β-d-glucose residues in its crystalline region and resisting hydrolysis[147]. Cellulases are a class of enzymes, including endoglucosidase that randomly cuts β-1,4-glycosidic bonds in releasing more ends., exoglucosidase that acts on ends to release cellulose dimers, and β-1,4-glucosidase that hydrolyzes dimers to fermentable sugars and breaks down cellulose and polysaccharides. All three enzymes are needed for efficient sugar conversion from cellulose[148]. Cellulases are sourced from various organisms, such as *Caldicellulosiruptor bescii*, *Pseudoalteromonas sp.* ArcC09, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* FW2[147-151]. Fungi, particularly thermophilic filamentous species, possess advanced enzyme systems for cellulose degradation. For instance, a cysticercus from eastern Russia demonstrated potent cellulose-dissolving ability. Ascomycete species like *Xylella rickettsii*, widely used in industry, secrete highly active cellulases[152, 153]. Optimal conditions for cellulose conversion to fermentable sugars occur at mild conditions, with temperatures between 40 to 50 °C and a pH of around 4.5[154].

1.6 Cellulosic lactic acid fermentation

Lactic acid (LA) is a versatile biomolecule with significant industrial and biomedical applications, including foods, cosmetics, textiles and pharmaceuticals[155-157]. Economically feasible and sustainable challenges in lactic acid production demand cost-effective and sustainable raw materials with minimal toxicity of byproducts and contamination, inexpensive substrates, continuous production on a large scale at a rapid rate with minimal and complicated handling process, environmental improvement, and increased biomass value[158-161]. Lactic acid can be produced via chemical synthesis from hydrocarbon-based sources or microbial fermentation. Chemical methods yield a racemic form, while microbial fermentation yields a higher optical purity of desired lactic acid[162]. Industrial bioconversion technology favors fermentation, utilizing microbial strains under optimal conditions. Chemical synthesis involves hydrogen cyanide and acetaldehyde reaction, distillation, hydrolysis, esterification, and acid

addition. Other chemical methods include oxidation of carbon monoxide, sugar degradation, chloropropionic acid hydrolysis, and propylene oxidation[159]. Therefore, microbial fermentation offers benefits like organic waste utilization, environmental friendliness, reduced energy consumption, the ability to use cost-effective substrates, and the possibility of pure lactic acid production[163-167].

The utilization of lignocellulosic biomass as an alternative substrate for microbial fermentation is more attractive and considered globally due to its abundance, renewability, cost-effectiveness, non-competition with food crops, and high sugar content. Carbohydrates constitute only a small portion of dry matter but require pretreatment, leading to inhibitory compounds. These inhibitory compounds will cause negative impacts on cell growth and fermentation performance of lactic acid-producing microbes[167-169]. Therefore, detoxification methods are crucial before the fermentation to overcome these drawbacks and achieve optimal bioproduct conversion. Microbial susceptibility to contaminants and environmental conditions further impact lactic acid fermentation[170, 171].

Pediococcus acidilactici ZY271 is an L-lactic acid-producing bacteria developed by combining genetic engineering and adaptive evolution. *P. acidilactici* ZY271 poses the ability to co-utilize glucose and xylose in the lignocellulose hydrolysate simultaneously. Initial efforts involved the PP pathway reconstruction by integrating transketolase (*tkt*) and transaldolase (*tal*) genes that resulted in an engineered strain (*P. acidilactici* TY112-*Δpkt::*(*tkt_tal*)) with improved L-lactic acid generation and reduced acetic acid formation. Further modifications (*P. acidilactici* TY112-Δ*pkt::*(*tkt_tal*)-Δ*ackA2::xylAB*) lead to enhanced xylose conversion into L-lactic acid with minimal acetic acid production. A stabilized strain *P. acidilactici* ZY271, with increased cell growth, L-lactic acid production and reduced residual xylose after adapted over 66 d. The adapted strain exhibited superior performance in media with xylose as the sole carbon source or a mix of glucose and xylose, showcasing simultaneous glucose and xylose utilization without carbon catabolite repression and minimal acetic acid byproduct. *P. acidilactici* ZY271 could produce a high L-lactic acid titer of 130.8 ± 1.6 g/L from detoxified wheat straw hydrolysate containing mixed glucose and xylose. The productivity of L-lactic acid fermentation could reach 1.82 g/L/h, and conversion of xylose could reach 94.9%, which indicated the potential of the strain as high optical L-lactic-acid producing bacteria for industrial cellulose L-lactic acid biorefinery application[172].

Consolidated bioprocessing (CBP) offers a promising approach to convert lignocellulosic biomass into L-lactic acid using thermophilic cellulolytic/hemicellulolytic bacteria, *Caldicellulosiruptor sp.* DIB 104C. This method bypasses the need for externally added

enzymes. *Caldicellulosiruptor sp.* DIB104C demonstrated the capability of producing L-lactic acid from microcrystalline cellulose or lignocellulosic substrates without external addition of cellulolytic/hemicellulolytic enzymes. By adaptive evolution, the strain increased L-lactic acid accumulation from 6 to 70 g/L on microcrystalline cellulose. Evolved strains also displayed a maximum lactic acid production rate of 1.0 g/Lh^{-1} and a lactic acid ratio of 96 wt% in the total organic fermentation products that indicated potential for a commercially integrated production process from lignocellulose materials[173].

1.7 *Paecilomyces variotii*

Paecilomyces variotii is a versatile thermos-tolerant fungus discovered in various environments, including food products, soil, indoor settings, and clinical samples. It exhibits thermotolerance and can grow in low oxygen conditions, surviving heat treatment and often contaminating heat-treated fruit juices[174, 175]. A previous study revealed that *P. variotii* poses the capability of converting ferulic acid as part of the lignocellulose-derived inhibitors to 4-vinylguaiacol through nonoxidative decarboxylation via methoxyhydroquinone through oxidation and demethylation reactions, which is then further converted to vanillin[176]. While in a direct transformation of ferulic acid to vanillin, *P. variotii* predominantly transforms vanillin to vanillic acid by oxidation to vanillyl alcohol. Vanillyl alcohol by reduction is potentially a means of mitigating the toxicity of vanillin. This study indicated the potential of this strain in degrading phenolic compounds generated from lignocellulose biomass pretreatment.

Furthermore, *P. variotii* was known for its applications in reducing cyclic terpenoids and aromatic aldehydes and detoxifying residual waters and agricultural effluents[177]. In particular, it demonstrated the capability to degrade various organic acids, such as oxalic acid, fumaric acid, citric acid and succinic acid, with oxalic acid selected as a promising pretreatment catalyst due to its biodegradability. Research on *P. variotii* FN89 also revealed its effectiveness in simultaneously detoxifying inhibitors such as acetic acid, HMF, furfural, and other phenolic compounds, degrading oxalic acid, and preserving fermentable sugars. The strain indicated its ability to degrade oxalic acid and other inhibitors within 48 h, with only vanillin taking 60 h. The inhibitors are converted into intermediate metabolites and drained into central metabolic pathways without residues[178].

P. variotii is also renowned for its biotechnological applications, including enzyme production and synthesis of novel compounds. Notably, it produces Pekilo® biomass, a single-cell protein used in animal feed production[179]. Its ligninolytic enzymes enable the conversion of complex growth substances like cellulose into value-added products such as bioethanol.

Additionally, its lipolytic enzymes facilitate the hydrolysis of oils and fats into biodiesel fuel[180, 181].

1.8 Adaptive laboratory evolution

Natural evolution is the process in which the biosphere teems with diverse life forms. It involves species undergoing mutations and recombination that lead to natural selection and favor the robust species with beneficial phenotypes for survival and reproduction[182]. Adaptive laboratory evolution (ALE) is the application of natural evolution principles in a controlled laboratory setting. It involves directing natural selection toward a chosen environment with desired conditions, promoting the accumulation of beneficial mutations while purging deleterious ones[183]. In adaptive laboratory evolution experiments, the emergence of beneficial mutations is increased due to applied selection as genetic dynamics in the population[184, 185]. Clonal interference, where multiple beneficial mutations compete simultaneously, is observed in asexual organisms. Sexual reproduction reduces this interference, and sexual recombination results in removing deleterious mutations[184, 186, 187].

The strength of applied selective pressure in adaptive laboratory evolution experiments can vary from weak to strong. Weak selection sustains genetic diversity and explores more mutational paths, while strong selection may lead to the survival of rare mutations and generate unique phenotypes. The duration of adaptive laboratory evolution experiments should be sufficient for achieving specific fitness. Prolonging the experiment beyond a certain point may be meaningless as populations tend to reach an adaptive plateau[188, 189]. Adaptive laboratory evolution experiments are performed mainly on microorganisms due to advantages such as short generation time, large population size, reproducibility, and relatively small genomes. Microbial ALE involves long-term cultivation under specific conditions to achieve desired traits[190-192]. General techniques for adaptive laboratory evolution include serial batch cultivations in shake flasks, chemostats, and turbidostats. Automation aims to consolidate multiple independent versions of adaptive laboratory evolution experiments, prone to drawbacks associated with manual procedures, as depicted in Figure 3[193]. The microbial evolutionary experiment represents an innovative approach that enriches our comprehension of how natural selection can prompt optimal genetic alterations and cellular adaptations in microbes to ensure survival under selective pressures. In addition, adaptive laboratory evolution (ALE) has emerged as a well-established and potent tool for biotechnologists to enhance desired strains for specific phenotypic fitness and investigate evolutionary behaviors that underlie survival during the adaptation process. ALE holds promise in bolstering the robustness of

microbial populations, thereby improving performance across various facets of industrial biotechnology. This thorough examination sheds light on the mechanisms of adaptation to stressful environments, elucidating effective strategies for overcoming limitations that may arise when imposing particular selective pressures on microbial populations.

Yeast strains' tolerance has been improved through multiple adaptive laboratory evolution experiments, particularly *Saccharomyces cerevisiae*, to inhibitors found in lignocellulosic biomass hydrolysates. Various stressors, including furfural, HMF, and acetic acid, have been employed to induce tolerance in yeast populations through adaptive laboratory evolution. Sugarcane bagasse hydrolysates were used in a previous study to achieve notable enhancements in ethanol productivity in *S. cerevisiae* strains[194]. In addition, a furfural-tolerant yeast strain was developed through adaptive laboratory evolution (ALE) using lignocellulose hydrolysates[195, 196]. The evolutionary process strategy utilized a cocktail of inhibitors in spruce biomass hydrolysates to enhance sugar consumption and ethanol productivity in yeast strains[197]. Another adaptive evolution strategy employed the visualizing evolution in real-time (VERT) method to produce *S. cerevisiae* strains tolerant to lignocellulosic biomass hydrolysates and revealed target molecular mechanisms involved in adaptation[198]. Evolutionary adaptation aimed to increase yeast tolerance to phenolics in corncob residues and resulted in strains with higher sugar consumption and ethanol productivity[199]. Evolutionary adaptation successfully improved *S. cerevisiae* tolerance to inhibitors derived from lignocellulosic biomass through ALE experiments[200, 201]. In addition, chemical mutagenesis was coupled with adaptive laboratory evolution to enhance inhibitor tolerance in a recombinant xylose-utilizing *S. cerevisiae* strain[202]. A separate study subjected a genetically modified *Zymomonas mobilis* strain to adaptive laboratory evolution, resulting in improved tolerance to inhibitors found in corn stover hydrolysates[203]. Notably, the evolved strain exhibited increased cell viability, desired product yield, and xylose uptake compared to the initial strain, with notable genetic alterations observed in the promoter region between specific genes[193]. These studies demonstrate the potential of adaptive laboratory evolution in developing microbial strains with enhanced tolerance to inhibitors for efficient bioproduct production from lignocellulosic biomass.

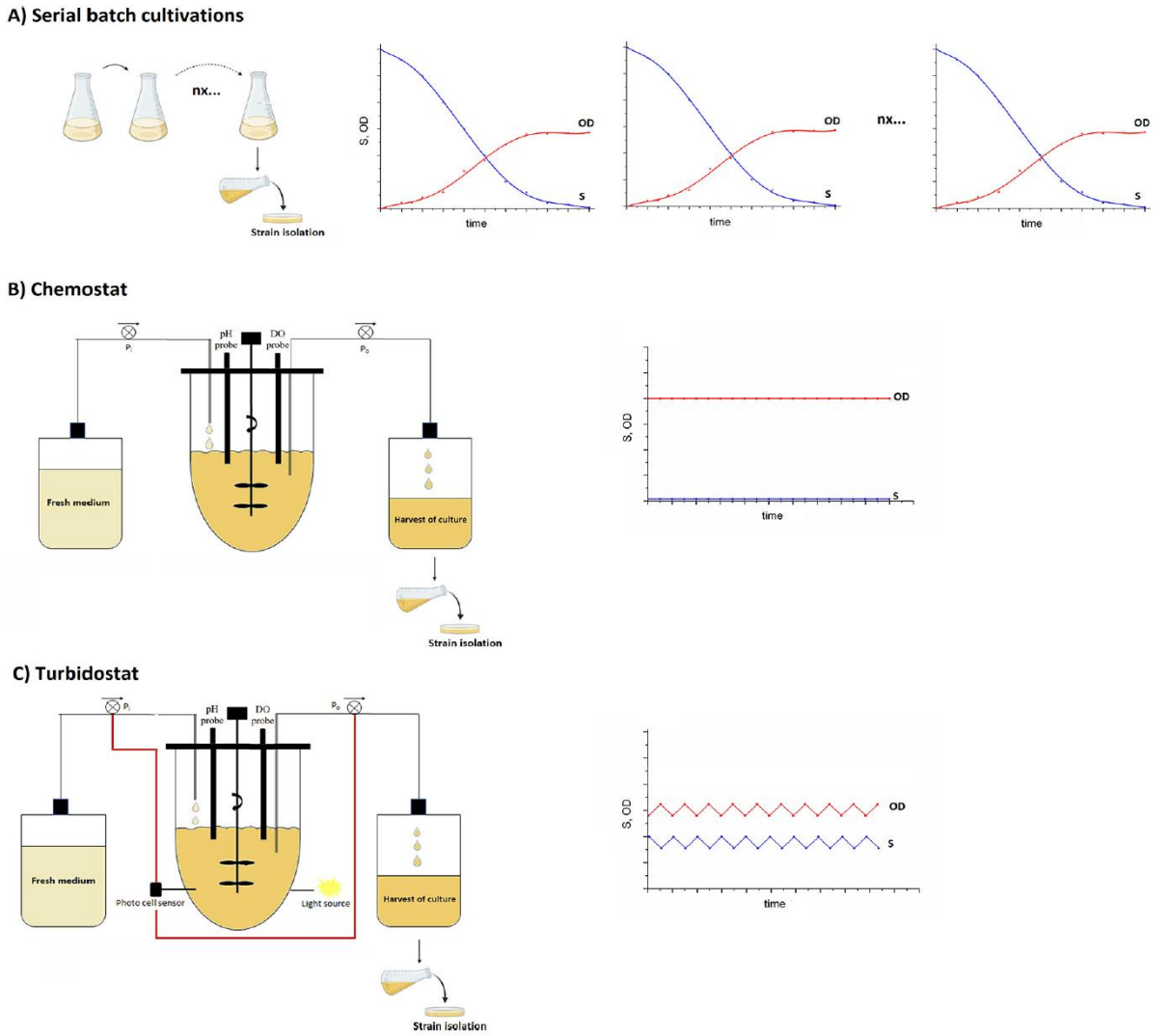


Figure 3 Brief scheme of adaptive laboratory evolution methods[175].

(A) Serial batch cultivation: Conducted in shaking flasks, growth conditions fluctuate as the concentration of the limiting growth factor (S) changes during microbial growth. In each adaptation cycle, the stress factor remains constant or increases. (B) Chemostat continuous culture: Carried out in a bioreactor, growth conditions are maintained constant throughout microbial growth. Steady-state conditions are achieved by controlling the limiting growth factor, and evolution occurs under nutrient limitation. The stress factor, if different from the limiting nutrient, remains constant or increases during evolution. (C) Turbitostat continuous culture: Also performed in a bioreactor, growth conditions remain almost constant during microbial growth. Steady-state conditions are controlled by the optical density (cell density) in the reactor, and evolution occurs under balanced growth conditions. The stress factor is kept constant or increasing during evolution.

1.9 Research objectives and significances

The lignocellulose biomass utilization for producing green materials is one of the emerging research fields to replace petrochemical-based materials. The rigid structure of the plant cell wall needs to be destroyed by a pretreatment process to obtain the fermentable sugar from lignocellulose. Dry dilute acid pretreatment (DDAP) is a promising approach for

maintaining high solid loadings without generating free water. In contrast, the approach could retain all the inhibitors generated during the process at a very high level in the pretreated feedstock. In addition, the high solid loadings will be implemented into hydrolysis to obtain high fermentable sugar, resulting in high inhibitors containing hydrolysate. These high inhibitors containing hydrolysates become the main drawback for microbial fermentation by inhibiting cell growth and decreasing the fermentation productivity. The detoxification step is a crucial approach to degrade all the inhibitors in the hydrolysate.

Biodetoxification is a promising approach to degrade the inhibitors in the hydrolysate without generating toxic compounds in the environment. The newly isolated biodetoxification strain, *P. variotii* FN89, is a potential strain due to its ability to degrade inhibitors in the hydrolysate. However, high inhibitors containing hydrolysates, such as wheat straw and corncob hydrolysates, could cause severe inhibition to the cell growth and reduction of the detoxification performance. Improving the biodetoxification performance is needed to overcome this limitation. First, the current strain, *P. variotii* FN89, can be developed to enhance its robustness toward high inhibitor concentration through adaptive laboratory evolution. Second, a new isolate from corncob hydrolysate with high concentration of acetic acid and furfural was assayed the ability of biodetoxification.

In this study, we employed an adaptive evolution strategy to enhance tolerance of *P. variotii* FN89 against high inhibitors containing lignocellulose hydrolysates, including acetic acid, HMF, and furfural for effective and efficient biological detoxification process for cellulosic L-lactic acid biorefinery production. In the second strategy, we isolated a tolerant strain as a potential detoxification strain to detoxify corncob residue hydrolysate and its utilization for cellulosic L-lactic acid biorefinery production. Details of research to achieve the above goals in this study are as follows:

1. Strategy for tolerance enhancement of biodetoxification strain *P. variotii* FN89 to high inhibitor concentration by adaptive laboratory evolution in solid and submerged cultivation for subsequent cellulosic L-lactic acid production.
2. Isolation, identification, and characterization of a tolerant strain *P. variotii* CC004 to high inhibitor containing corncob residue hydrolysate for cellulosic L-lactic acid production.

Chapter 2 Inhibitor Tolerance Enhancement and L-lactic Acid Fermentation Evaluation of An adapted *Paecilomlyces variotii*

2.1 Background

The pretreatment of lignocellulose is crucial for lignocellulose-based biorefinery, but the existing methods have drawbacks such as high water and steam usage, wastewater generation and toxin production. Conventional pretreatment involves saturating lignocellulose materials in a large volume of dilute acid solution before subjecting them to high temperatures. A recent innovation, dry dilute acid pretreatment (DDAP), minimizes dilute acid usage to reduce wastewater production. The dry dilute acid pretreatment method with low steam usage significantly reduces the hot steam consumption, leading to a sharp reduction in energy consumption. Furthermore, the dry pretreatment method does not release aqueous acid-containing water during the pretreatment operation, reducing the burden of wastewater treatment. The dry process is involved in the process in which half of the pretreated materials are dry solids, making the pretreatment a dry-to-dry process. This process allows for feeding with dry lignocellulose feedstock, resulting in a dry pretreated product without any wastewater generation. High solids loading in dry pretreated products allows high solids loading (30%, w/w) in the subsequent saccharification and fermentation process and reduces water usage[204-206].

Dilute acid pretreatment of lignocellulosic enhances cellulose accessibility for enzymatic digestion by hydrolyzing hemicellulose, but it also generates inhibitory compounds that are toxic to fermentative microorganisms. Chemical depolymerization of hemicellulose yields various sugars and inhibitors, including furan derivatives, phenolic compounds and acetic acid[57, 207]. High solid ratio conservation in dry dilute acid pretreatment results in higher conservation of inhibitors generated during the process. Subsequent high solid loading during the saccharification process results in high inhibitors containing lignocellulose hydrolysate that become the drawbacks for microbial fermentation. In this term, a detoxification process is a crucial step before the fermentation process. High inhibitors containing hydrolysate will cause inhibition to the current biodetoxification strain and a further slow removal rate of toxins, significantly limiting its practical applications. To overcome these main hurdles, developing the robustness of the current detoxification strain to high concentrations of inhibitors needs to be addressed.

Generally, the concentration of acetic acid in lignocellulose hydrolysate typically ranges from 10 to 20 g/L[208, 209]. Additionally, furfural is present at levels between 0.3 to 8.5 g/L, while

HMF ranges from 0.1 to 0.7 g/L[210]. Undissociated acetic acid can permeate the cell membrane through passive diffusion and dissociate into proton and acetate ions within the cytosol. This process leads to a decrease in intracellular pH that potentially reaches levels intolerable for the cell and ultimately results in metabolic inhibition and cell death[211, 212]. Furfural presence in the hydrolysate could reduce xylose yield during pre-hydrolysis, induce single-strand breaks in double-stranded DNA, inhibit protein and nucleic acid biosynthesis, and significantly impair yeast respiration[213]. HMF can disrupt microbial macromolecule function and transformation, impede microorganism growth, alter microbial community structure in anaerobic sludge and hinder biofuel production in anaerobic fermentation[214]. On the other hand, lignin-derived phenolics are found in hydrolysates as well, albeit at concentrations typically 10 to 100 times lower than those of weak acids and furan aldehydes[215] and some phenolic aldehydes could be degraded by detoxification strain, such as *P. variotii* FN89[178].

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[216]. Solid substrate cultivation facilitates direct interaction between the microbial and the growth substrate under conditions with low water activity[217]. This study aimed to develop a robust strain of *P. variotii* FN89 through the adaptive laboratory evolution strategy to boost the tolerance of parental strain against high major inhibitors (acetic acid, HMF, furfural) containing lignocellulose hydrolysate. We performed adaptive laboratory evolution to enhance tolerance of *P. variotii* FN89 to high concentrations of lignocellulose inhibitors (acetic acid, HMF, furfural) by serial batch transferring in solid PDA medium and liquid synthetic medium (SM) till 70 transfers to induce consecutive tolerance. The biodetoxification performance evaluations were performed in a 3 L bioreactor using inhibitors containing synthetic medium and wheat straw hydrolysate. The transcriptional level of target genes involved in acetate and furan aldehydes metabolism was analyzed using qRT-PCR (real-time quantitative PCR).

2.2 Materials and methods

2.2.1 Strain and culture

Biological detoxification strain *P. variotii* FN89 (CGMCC 17665) served as the parental strain for adaptive laboratory evolution conducted at a constant temperature of 37 °C through two distinct methods. The first method utilized potato-dextrose-agar (PDA) gel in Petri dishes

(200 g/L potato infusion, 20 g/L glucose and 20 g/L agar). The second method employed a liquid synthetic medium (SM) (2 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L yeast extract (YE), 0.5 g/L $CaCl_2$ and 15 g/L glucose), which also functioned as the seed culture medium for *P. Variotii*.

For seed culture preparation, frozen stock *P. variotii* FN89 was performed on a PDA agar plate at 37 °C for 3-4 d to generate spores necessary for seed culture. Spores were collected by washing the plate with 10 mL of 0.05% (w/v) Tween 80 solution. Subsequently, the spores were scraped gently using a sterilized cell spreader to obtain a spore suspension. This suspension was then transferred into a 100 mL flask containing seed culture medium (synthetic medium with 20 g/L glucose) and incubated at 37 °C with 300 rpm agitation for 24 h.

The lactic acid-producing strain *P. acidilactici* ZY271 (CGMCC 13611) served as the fermentative strain. The seed culture medium comprised Man-Rogosa-Sharp (MRS) medium with 20 g/L glucose, 10 g/L tryptone, 10 g/L Yeast Extract, 5 g/L sodium acetate, 2 g/L ammonium citrate dibasic, 2.6 g/L $KH_2PO_4 \cdot 3H_2O$, 0.58 g/L $MgSO_4 \cdot 7H_2O$, and 0.25 g/L $MnSO_4 \cdot H_2O$. Activation of frozen stock *P. acidilactici* ZY271 involved inoculation into 20 mL MRS medium in a 100 mL flask and cultivation at 42 °C with 150 rpm agitation for 12 h. Subsequently, seed culture preparation was prepared by transferring 10 mL of the broth into a 500 mL flask containing 100 mL of MRS medium supplemented with glucoamylase at a 1% (w/w) mass ratio to prevent cell flocculation by breaking polysaccharide links among cell aggregations during cultivation. The seed culture was cultivated at 42 °C with 150 rpm agitation for 8 h.

2.2.2 Raw materials

Wheat straw was selected as the feedstock, collected from Nanyang City, Henan province, China, in the fall of 2022. The feedstock underwent a series of processing steps for industrial-scale biorefining. Initially, the feedstock was coarsely cut into pieces and washed to eliminate field impurities. Subsequently, the chopped feedstock would undergo an air-drying and milling process to achieve a particle size that can go through a sieve with a diameter of 10 mm. An essential de-dusting step was subjected to the pre-handling stage according to mechanical approaches as reported by the National Renewable Energy Laboratory (NREL) to remove a portion of dust[190]. However, for laboratory bench scale operations, employing a mechanical approach has proven impractical, leading us to opt for water washing as an alternative solution.

2.2.3 Enzyme and reagents

In this study, the commercial cellulase enzyme Cellic CTec 2.0 was utilized for the saccharification process, procured from Novozymes, China. The enzyme specifications for filter paper activity, cellobiase activity, and protein content were quantified at 203 FPU/mL, 4900 CBU/mL, and 87.3 mg/mL, respectively, following established protocols (Bradford, 1976; Ghose, 1987; Adney & Baker, 1996). Yeast extract (LP0021B) and peptone in reagent grade were purchased from Oxoid. Glucose, sulfuric acid, and other chemicals in reagent grade were purchased from Shanghai Titan Technology Co., Ltd.

2.2.4 Adaptive evolution biodetoxification stain for the enhanced inhibitor tolerance

The adaptive laboratory evolution (ALE) of *P. variotii* FN89 involved with PDA agar plates and synthetic liquid media with increasing inhibitor concentrations.

ALE on PDA agar plates:

For adaptive laboratory evolution in the 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 a 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.

ALE in synthetic liquid media:

Seed culture preparation followed by inoculating of 10% (v/v) seed culture into 50 mL synthetic liquid medium with initial inhibitor concentrations of 5 g/L sodium acetate, 0.5 g/L HMF and 0.5 g/L furfural and incubating at 37 °C for 72 h with 300 rpm agitation. Serial transferring of 10% cells was carried out by transferring 10% (v/v) of the cell suspension from previous cultivation to fresh media with incrementally higher inhibitor concentrations until the final concentrations of 15 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural. The samples were collected at each transfer for dry cell weight, acetic acid, HMF, and furfural residual analysis. The serial batch transferring was repeated until the 70^{th} transfer.

2.2.5 Wheat straw pretreatment

The wheat straw underwent a dry dilute acid pretreatment method, following established procedures[185-186]. The pretreated wheat straw had a moisture content of 50.65%. Subsequently, its composition was analyzed following NREL protocols[191-192], revealing 31.67% cellulose,

4.46% xylan, 8.94 ± 0.06 mg/g furfural, 3.62 ± 0.01 mg/g HMF, and 30.46 ± 0.23 mg/g acetic acid, all reported on a dry weight basis (w/w).

2.2.6 Wheat straw hydrolysate preparation

The pretreated wheat straw underwent hydrolysis in a 5 L bioreactor with a helical ribbon impeller at 50 °C through a fed-batch process within 12 h. The cellulase enzyme Cellic CTec 2.0 was added to the bioreactor at 4 mg of cellulase proteins per gram of dry pretreated wheat straw. The final solids loading was 25% (w/w), and the pH of pretreated wheat straw was adjusted to approximately 5.5 using 25% calcium hydroxide before loading into a 5 L bioreactor. Celtic CTec 2.0 cellulase enzyme was added to the bioreactor when the temperature reached 50 °C to maintain enzyme activity. Hydrolysis conditions were set at 50 °C under 200 rpm agitation for 12 h.

2.2.7 Biodetoxification performance evaluation

Comparative Evaluation in Synthetic Medium

In a 3 L bioreactor, a synthetic medium with 15 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural, without pH adjustment, was used for the first comparative evaluation. Biological detoxification involved inoculating a 10% seed culture of *P. variotii* FN89 and evolved strains. The conditions included 500 rpm agitation at 37 °C for 48 h with 1.0 vvm aeration. Periodic samples were collected to analyze inhibitor and sugar residual concentrations during detoxification.

Comparative Evaluation in Wheat Straw Hydrolysate

Another comparative evaluation was conducted in a 3 L bioreactor using undetoxified 25% (w/w) wheat straw hydrolysate. The detoxification was initiated by inoculating a 10% seed culture of P. variotii FN89 and evolved strains. Detoxification conditions for wheat straw hydrolysate included 750 rpm agitation at 37 °C and 1.0 vvm aeration. Periodic samples were collected to analyze inhibitor and sugar residual concentrations during detoxification.

2.2.8 L-lactic acid fermentation performance evaluation

Detoxified wheat straw hydrolysate by each strain underwent lactic acid fermentation directly in a 3 L bioreactor. The fermentation process involved inoculating 10% of *P. acidilactici* ZY271's seed culture into detoxified hydrolysate and adding nutritional salts (15 g/L YE, 10 g/L tryptone, 2 g/L ammonium citrate dibasic and 0.25 g/L $MnSO_4 \cdot H_2O$). Fermentation occurred in anaerobic conditions at 42 °C and 300 rpm for 60 h without aeration. The pH during fermentation was maintained at 5.5 using 25% calcium hydroxide. Periodic samples were

collected to analyze lactic acid and sugar residual concentrations during fermentation.

2.2.9 RNA extraction and transcriptional analysis

Cultivation of the evolved strains and parental strain *P. variotii* FN89 were grown in a synthetic medium with 20 g/L glucose and then cultivated at 37 °C for 24 h. Following cultivation, the cell suspension underwent centrifugation at 12,000 rpm for 5 minutes at 4 °C, followed by triple washing with Double distilled water to collect the cells and stored at −80 °C freezer for subsequent RNA extraction. The frozen cell mass was finely powdered using liquid nitrogen to extract total RNA, then subjected to the extraction step using Trizol reagent (RNAiso Plus, TAKARA, Otsu, Japan). The obtained RNA samples were assessed for their integrity and quantity based on the OD_{260}/OD_{280} ratio using a DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) and then subjected to reverse transcription reactions by using the ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (Toyobo, Osaka, Japan). The cDNA amplification was performed as following procedure: incubation at 37 °C for 15 min, followed by incubation at 50 °C for 5 min, heat treatment at 98 °C for 5 min, and storage of the reacted solution at 4 °C. Real-time quantitative PCR (qRT-PCR) reaction was conducted using SYBR Green Realtime PCR Master Mix kit (Toyobo, Osaka, Japan) on a BioRad CFX 96 (Hercules, CA, USA) with the following protocols: initial denaturation at 95 °C for 1 minute, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s, and extension at 72 °C for 30 s, concluding with a final melting curve analysis by heating from 65 to 95 °C with a rate of 0.5 °C per 5 s. Primers for qRT-PCR analysis are presented in Table 3. The 18S rRNA-ITS gene was employed as an internal control to normalize total RNA quantity differences. To quantify gene transcription levels, the $2^{\wedge-\Delta\Delta C}$ formula was used, with genes exhibiting a fold change (FC) of ≥ 2.0 or ≤ 0.5 considered significantly up-regulated or down-regulated, respectively.

Table 3 Primers for acetic acid and furan conversion-relating genes

Primers	**Sequences (5'-3')**
ACS-C8Q69DRAFT_68314	Forward: TGAGAAGGTCGGAAAGGAA
	Reverse: CTCGGTTTGCCAGTAGGTC
ACH1-C8Q69DRAFT_44388	Forward: CGTATGCTGAACGGTCTCG
	Reverse: GTCGGTCTTGCTGGGTCT
ACKA-C8Q69DRAFT_472720	Forward: TCTGAGGAAGCGAGTTGT

	Reverse: ACCTGTTCATCCGTCTGG
CS1-C8Q69DRAFT_499756	Forward: TCGCTCCTGGTGTTCTGA
	Reverse: CATTCGGGTGAGGGTTCT
ALDH3-C8Q69DRAFT_157210	Forward: TCCGAGTTCCCTTTGGTG
	Reverse: ATTGCGGTTCGTGCTGAT
ADH-C8Q69DRAFT_396683	Forward: TCTGTACGGCGTCGAATG
	Reverse: GCTTCTGTCCGATTACCAAC
ADHV-C8Q69DRAFT_510087	Forward: TCCTACGTCGGTAATCGC
	Reverse: TCATAAACCTTCGGCAAAT
ADH8-C8Q69DRAFT_469617	Forward: CTTCACCGCACCATACTTT
	Reverse: TCGGCAACGACATAGATTTT
ARI-C8Q69DRAFT_486534	Forward: CCATGATCCGCTACCACT
	Reverse: GAAGGGACATCGTCACTCT
AKR-C8Q69DRAFT_334300	Forward: CACAAGTCGCACTCGGTAA
	Reverse: AGAATGAAATCGCCCTCC
ALDH-C8Q69DRAFT_482952	Forward: TCTGTTTGCCGGGATTGT
	Reverse: TTCATTTCACGCTAGTTTCA
FAD-OR-C8Q69DRAFT_471500	Forward: CAACAATCCGCAGCACTC
	Reverse: AGGGACGGAATCAATCAC
HmfG-C8Q69DRAFT_246470	Forward: GTTCGCCTGTAGAAGTGG
	Reverse: ATGGCTCATCAAGAGGTTGG
GMC-C8Q69DRAFT_492106	Forward: GCGAGGTTTCCAGCAGTG
	Reverse: TGACGTATTCCGCCCACT
GMC1-C8Q69DRAFT_12690	Forward: CCTCGCAGTCCAGTCCTT
	Reverse: CGCATCGGTGGTGTAGTT
GDH-C8Q69DRAFT_511100	Forward: CCCGACACCTTCTCCAGT
	Reverse: CCGTACCTCATGCCATCC
MFS-C8Q69DRAFT_453425	Forward: AGACGGGCTGGATAGGAT
	Reverse: CGGCGTCAGGAACAGATA

2.2.10 Analytical methods

Analysis of glucose, xylose, lactic acid, acetic acid, 5-hydroxymethylfurfural (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 set at 65 °C, with a mobile phase comprising a 5 mM H_2SO_4 solution flowing at a rate of 0.6 mL/min.

Dry cell weight (DCW) was determined by collecting cell pellets from cell suspension by filtering all the liquid, rinsing them with distilled water, and subsequently drying at 105 °C overnight.

2.3 Results and discussion

2.3.1 Adaptive evolution of *P. variotii* FN89 under high inhibitor cocktail concentration

Developing robust detoxification strains capable of tolerating a broad range of inhibitor concentrations is imperative for enhancing lignocellulose-based biorefinery processes. To improve the tolerability of *P. variotii* FN89 to extremely high inhibitor concentrations, we conducted adaptive evolution on the parental strain *P. variotii* FN89 using two methods, including solid-surface culture on PDA agar plates (Figure 4) and submerged liquid culture in synthetic medium (Figure 5). These cultures were incrementally exposed to escalating concentrations of acetic acid, HMF, and furfural gradually until reaching the maximum levels typically encountered in high-severity pretreatment operations. 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[210]. 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.

Figure 4 showed the adaptive evolution on the PDA agar plate from an initial concentration of 5 g/L sodium acetate, 0.5 g/L HMF and 0.5 g/L furfural, and then gradually increased to 15 g/L, 1.5 g/L and 0.5 g/L, respectively in five stages (equivalent to 70 transfers and 253 d). When growing stable (spores germinated with visible mycelia within 3 d) in one phase, the cells were transferred to the next phase with higher inhibitor concentrations. The colony size of the cells on the PDA gel was transformed into a smaller colony starting at 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 transformed into a deep yellowish-green from green starting at the fourth phase when the inhibitors were elevated 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 indicated a stable state without further observable changes in morphology and after 70 transfers, thus suggesting the cells reached their threshold, and the evolved strain was defined as *P. variotii* AC70.

Figure 5 demonstrated the growth profile of *P. variotii* FN89 during adaptive laboratory

evolution in a liquid synthetic medium supplemented with an increasing concentration of inhibitors cocktail. Initial transfer indicated a short period of fluctuating cell growth, followed by higher acetic acid residual. However, after continuous six to eight transfers, the strain demonstrated stable cell density and acetic acid conversion. Interestingly, we observed the strain had a preferential to degrade HMF and furfural over acetic acid in each transfer during cultivation, indicating the almost complete reduction of HMF and furfural during the evolutionary process. In contrast, higher acetic acid conversion was initiated after achieving complete conversion of HMF and furfural. When the strain was transferred into the synthetic medium containing 15 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural, the cells maintained stable cell growth, but acetic acid residual was higher and not stable during the ten initial transfers. The evolutionary process at the last phase was terminated and defined as *P. variotii* ZW70 after 70 transfers in 210 d, observed with the cell growth achieving a relatively stable stage (~ 4 g/L dry cell weight) followed by the residual acetic acid concentration below 2.5 g/L.

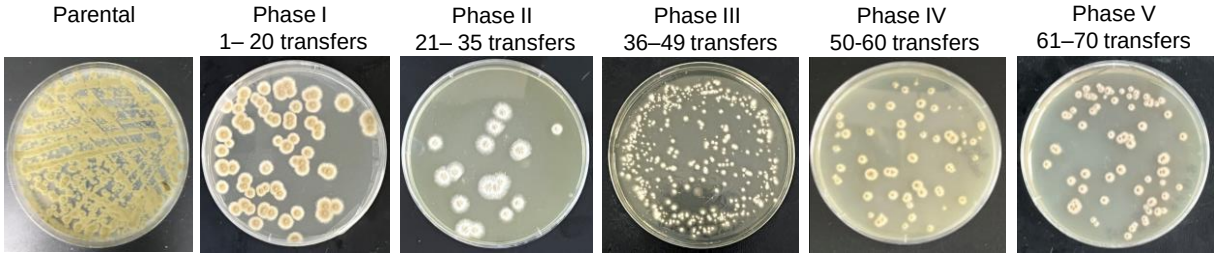


Figure 4 Morphology changes of *P. variotii* FN89 colonies during adaptive laboratory evolution on PDA agar plate. Serial transfer of the cell into fresh agar plate medium containing inhibitors cocktail was repeated every 3-4 days. 1st phase: PDA agar plate enriched with 5 g/L sodium acetate, 0.5 g/L HMF and 0.5 g/L furfural; 2nd phase: PDA agar plate enriched with 7 g/L sodium acetate, 1.0 g/L HMF and 0.5 g/L furfural; 3rd phase: PDA agar plate enriched with 9 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural; 4th phase: PDA agar plate enriched with 11 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural; 5th phase: PDA agar plate enriched with 15 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural. The whole evolutionary process lasted 70 generations for 253 d.

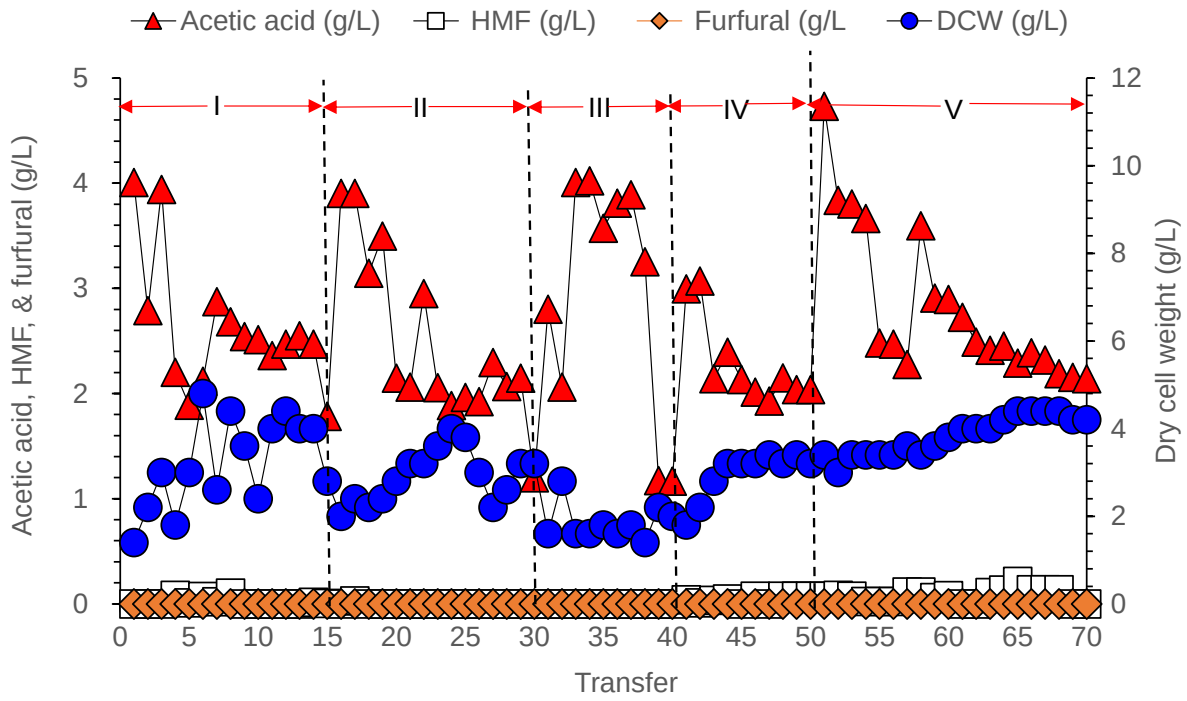


Figure 5 Profiling of dry cell weight (DCW), acetic acid, HMF and furfural residual during adaptive evolution of *P. variotii* FN89 in liquid synthetic medium containing 15 g/L glucose. Serial transfer of 10% (v/v) of the cell culture into a fresh medium containing inhibitors cocktail was repeated every 72 h. The initial pH was adjusted to 4.5 using HCl. Five phases of ALE were performed: 1st phase: synthetic medium containing 5 g/L of sodium acetate, 0.5 g/L of HMF, and 0.5 g/L furfural; 2nd phase: synthetic medium containing 7 g/L of sodium acetate, 1 g/L of HMF and 0.5 g/L furfural; 3rd phase: synthetic medium containing 9 g/L of sodium acetate, 1.5 g/L of HMF and 0.5 g/L furfural; 4th phase: synthetic medium containing 11 g/L of sodium acetate, 1.5 g/L of HMF and 0.5 g/L furfural and 5th phase: synthetic medium containing 15 g/L of sodium acetate, 1.5 g/L of HMF and 0.5 g/L furfural. The whole adaptive evolution had lasted for 70 generations.

The morphology of evolved strains *P. variotii* AC70 and *P. variotii* ZW70 was observed by cultivating them in PDA agar plates with and without inhibitors of 15 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural. Compared with the parental strain FN89, both evolved strains exhibited small colony size transformation growing on the PDA agar plate with and without inhibitors cocktail (Figure 6). In addition, it is noteworthy that the evolved strains *P. variotii* AC70 and *P. variotii* ZW70 exhibited an undulated margin at the colony center as the growth response, and they produced reduced conidiophores when exposed to stressful environmental conditions. Additionally, the conidiophores color of evolved strain *P. variotii* AC70 and *P. variotii* ZW70 transformed into deeper green and yellowish-green color, respectively. There's a possible explanation that the small colony size transformation of both evolved strains might be associated with aneuploidy as the response to environmental stress during adaptive evolution. We assumed that small cell size caused by aneuploidy could trigger stress responses aimed at

metabolizing inhibitors and preventing cellular damage induced by the stress. Aneuploidy has been reported previously in inducing contrasting effects, including disrupting protein abundances and impeding cell growth. In addition, aneuploidy could also simultaneously foster genetic diversity and facilitate rapid adaptation to stressful environments. The small cell transformation could be identified during evolutionary adaptation in a solid agar plate supplemented with a gradually increasing concentration of inhibitors cocktail (Figure 6). A previous study revealed that the robust strain *S. cerevisiae* to ethanol shared chromosome III aneuploidy[218]. Detail ploidy-level investigation in both evolved strains *P. variotii* AC70 and *P. variotii* ZW70 needs further investigation to provide a better understanding of the relationship between aneuploidy and strain robustness on *P. variotii*.

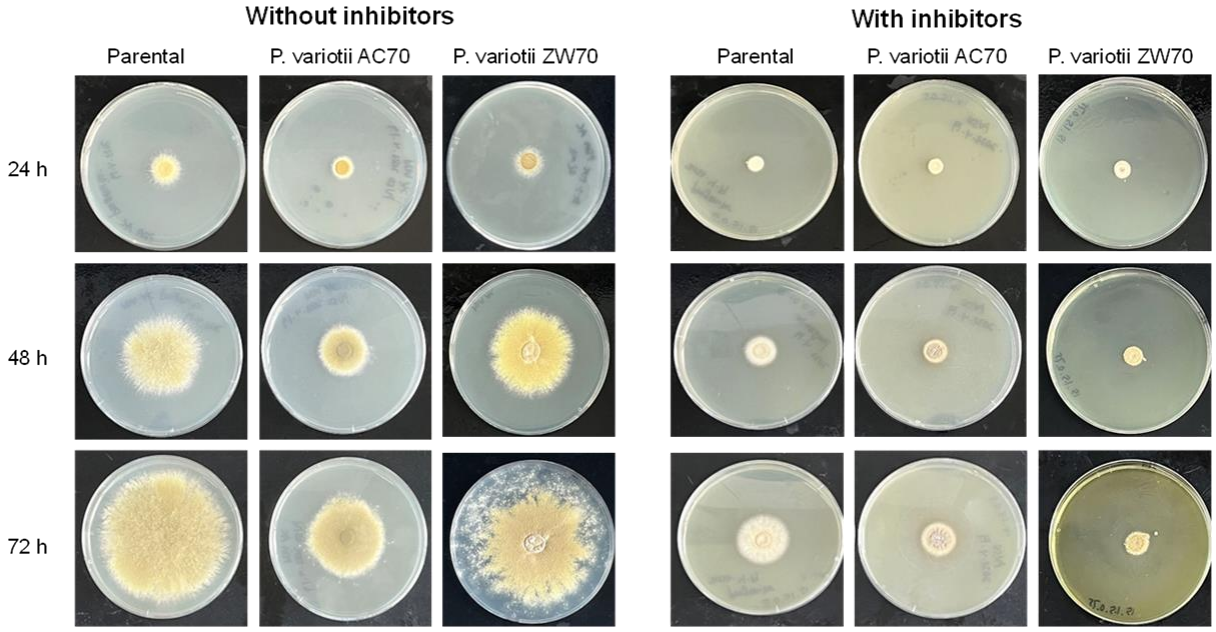


Figure 6 Morphology of the adaptively evolved strains *P. variotii* AC70, ZW70, and the parental strains on PDA plate without and with 15 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural within 72 h cultivation at 37 °C.

2.3.2 Evaluation of biodetoxification and lactic acid fermentation performance

For further characterization, we conducted a comparative evaluation of detoxification performance between the evolved strains *P. variotii* AC70 and *P. variotii* ZW70 and the progenitor strain FN89 in a 3 L bioreactor using synthetic medium supplemented with 15 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural. As depicted in Figure 7a, the two evolved strains *P. variotii* AC70 and *P. variotii* ZW70 demonstrated superior cell survival, with increased cell density from 2.186 g/L and 2.431 g/L to 4.147 g/L and 3.861 g/L after 72 h cultivation, respectively. In contrast, parental strain exhibited severe cell growth inhibition, as

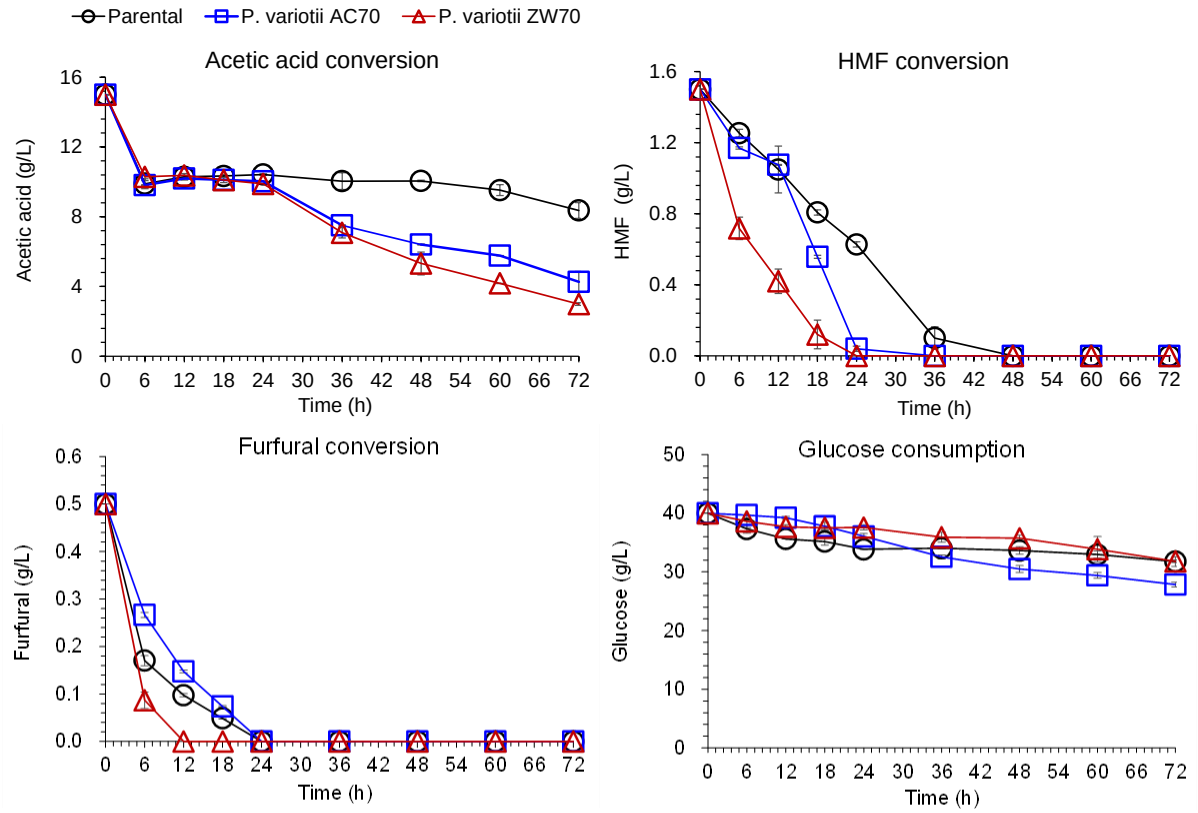
indicated by a decline in cell density from 1.716 to 1.144 g/L at 72 h during the detoxification. These results represented 3.62 and 3.37 times higher cell density, respectively, compared with parental strain *P. variotii* FN89. These results indicated the negative impact on cell growth due to the high concentration of inhibitors was evident in progenitor *P. variotii* FN89. However, evolved strains could maintain their growth capacity conferred through adaptive laboratory evolution in a stressful environment. Both evolved strains exhibited similar behavior in glucose and xylose consumption during the cultivation in which xylose was less utilized than glucose during cultivation. Such a decreased utilization of xylose might be due to the inhibitory effects of the inhibitors cocktail on xylose metabolism over glucose. In addition, evolved strain *P. variotii* AC70 and *P. variotii* ZW70 efficiently degraded 1.5 g/L HMF and 0.5 g/L furfural after 24 h, indicating prior utilization of these compounds as carbon sources during cultivation. Evolved strain *P. variotii* ZW70 demonstrated remarkable removal rates for HMF and furfural, outperforming parental strain FN89, and the evolved strain *P. variotii* AC70 exhibited a 16.72% better efficiency in furfural removal at 6 h compared to FN89 and a 45.78% faster removal rate of HMF at 18 h. On the other hand, evolved strain AC70 displayed a 39.16% higher removal rate of HMF after 24 h compared to parental strain *P. variotii* FN89. Notably, both evolved strains *P. variotii* AC70 and *P. variotii* ZW70 also showed a higher rate of acetic acid removal, which were 27.31% and 35.78% improvement, respectively, compared with the parental strain *P. variotii* FN89 after 72 h cultivation.

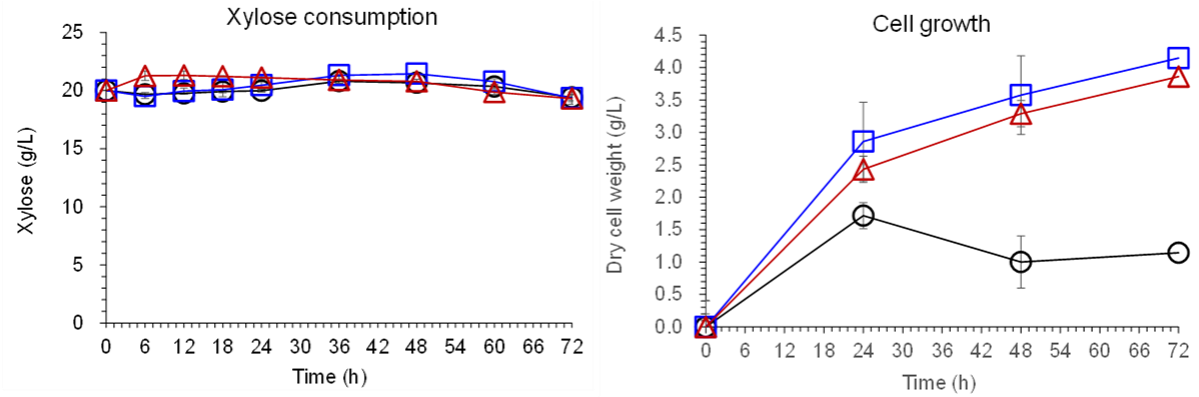
Subsequent evaluation of detoxification performance between the evolved and parental strains was then carried out in high inhibitors containing wheat straw hydrolysate. Based on the data illustrated in Figure 7b, we noticed similar glucose and xylose consumption patterns in both the evolved and parental strains while undergoing detoxification. Evolved strains *P. variotii* AC70 and *P. variotii* ZW70 demonstrated remarkable furfural degradation within a 6 h shorter time frame compared to parental strain FN89, which were improved by 23.55% and 25.04%, respectively after 18 h cultivation. Furthermore, both evolved strain *P. variotii* AC70 and *P. variotii* ZW70 also exhibited a 9 h shorter time for HMF conversion, indicating enhanced detoxification capabilities in furan aldehydes that were improved by 57.24% and 59.84%, respectively, better than the parental strain after 27 h cultivation. Both experiments of biodetoxification in synthetic medium and wheat straw hydrolysate revealed the complete conversion of furan aldehydes before acetic acid. In addition, evolved strain *P. variotii* AC70 and *P. variotii* ZW70 exhibited significantly faster acetic acid removal rates than the parental strain, which were 51.71% and 54.39% improvement. We observed a similar trend of

biodetoxification in synthetic medium and wheat straw hydrolysate, of which degradation of acetic acid was higher after achieving complete or almost complete degradation of furan aldehydes. The observed correlation between furfural degradation and accelerated acetic acid removal suggests a strategic approach to detoxification, prioritizing the removal of the most toxic compounds first. These results underscore the importance of understanding the detoxification mechanisms of *P. variotii* and optimizing them for enhanced biorefinery.

In summary, adaptive laboratory evolution strategies, such as those employed in this study, have proven effective in selecting improved phenotypes under defined conditions. This research adds to the development of robust detoxification strains that can effectively handle lignocellulose hydrolysate, making it a promising solution for improving industrial detoxification processes.

(a) Biodetoxification in synthetic medium





(b) Biodetoxification in 25% (w/w) wheat straw hydrolysate

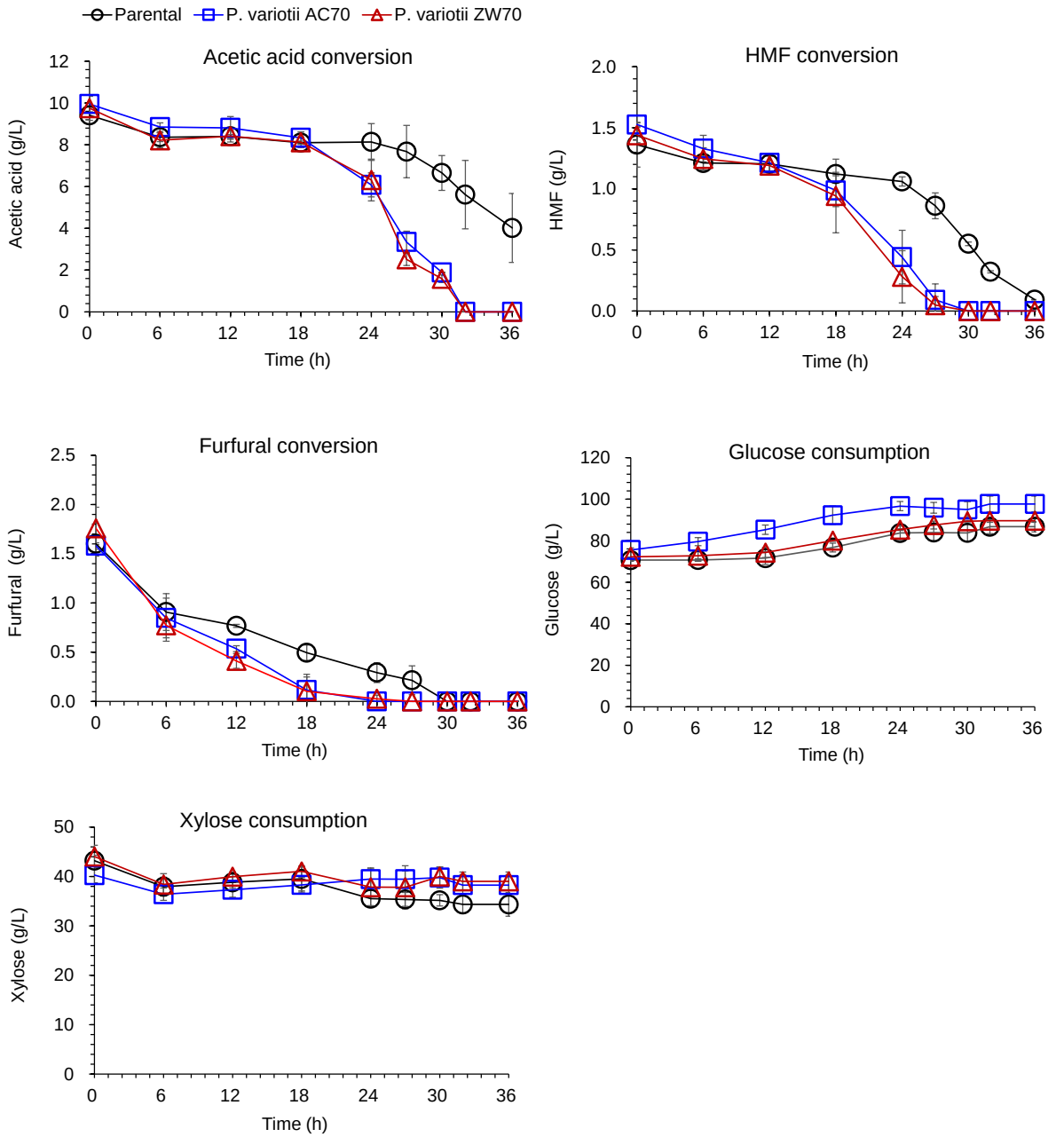


Figure 7 Biodetoxification evaluation in (a) synthetic medium supplemented with 15 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural and (b) high inhibitors containing wheat straw hydrolysate. The detoxification was

carried out in a 3 L bioreactor at 37 ºC with 750 rpm agitation and 1 vvm aeration. All data were obtained by two experiments and presented as mean ± SD.

The study aimed to analyze how bio-detoxified wheat straw hydrolysates impact the performance of L-lactic acid fermentation in *P. acidilactici* ZY271, so the fermentation evaluation was performed using biodetoxified hydrolysates. Figure 8 illustrated that detoxified wheat straw hydrolysate processed by evolved strains *P. variotii* AC70 and *P. variotii* ZW70 consistently exhibited higher lactic acid accumulation (110.05 ± 4.81 – 110.27 ± 2.18 g/L vs 90.49 ± 10.09 g/L), showing a 22% improvement compared with hydrolysate detoxified by parental strain. The lactic acid productivity of the detoxified wheat straw hydrolysate processed by evolved strains *P. variotii* AC70 and *P. variotii* ZW70 were 1.528 – 1.531 g/Lh^{-1} versus 1.256 g/Lh^{-1}, indicating a 22% higher compared to hydrolysate detoxified by parental strain. The possible explanation might be due to the higher residual acetic acid level (4.02 ± 1.65 g/L) of detoxified hydrolysate by parental strain at the initial fermentation, negatively impacting the fermentation productivity of *P. acidilactici* ZY271. Additionally, the sugar utilization rate of *P. acidilactici* ZY271 in detoxified hydrolysate by parental strain decreased significantly after 12 h of cultivation. Almost complete sugar utilization was achieved within 48 h of fermentation times for detoxified hydrolysate by evolved strains *P. variotii* AC70 and *P. variotii* ZW70, representing a 12 h shorter fermentation period than parental strain *P. variotii* FN89. This result indicates higher initial concentrations of acetic acid can impede the conversion rate of fermentable sugars to L-lactic acid by *P. acidilactici* ZY271.

Previous studies have reported that 5.3 g/L acetate can cause growth inhibition in *P. acidilactici*, resulting in a 4% decrease in cell growth, with further synergistic effects when acetate is combined with furfural. Mechanisms of *P. acidilactici* response to acetic acid involve the downregulation of glycolysis and gluconeogenesis pathways, pyruvate metabolism, and PTS, followed by increased fatty acid saturation and antioxidant enzyme activities[219]. Accumulation of acetic acid and lactic acid during lactic acid fermentation can lead to a decrease in intracellular pH, affecting the transmembrane pH gradient and causing growth inhibition due to impaired cellular functions and diverted energy towards pH maintenance rather than growth[212]. This condition ultimately results in slowed lactic acid production and potential disruption of cell membrane integrity[220]. In addition, when pentoses are present as substrates for lactic acid fermentation, microorganisms ferment pentoses hetero-fermentatively via the phosphoketolase (PK)-pathway, producing by-products such as acetic acid, which can reduce lactic acid yield[221]. This phenomenon could explain the higher initial concentration of

acetic acid in detoxified wheat straw hydrolysate by parental strain, resulting in lower lactic acid accumulation after 72 h of fermentation. During the cultivation process, the fermentable sugars present may get consumed to support the growth of cells.

In summary, the detoxification performance of evolved strains *P. variotii* AC70 and *P. variotii* ZW70 exhibited remarkable removal rates of major inhibitors present in wheat straw hydrolysates. Moreover, the detoxified hydrolysates processed by both evolved strains resulted in improved lactic acid accumulation. This study underscores the development of detoxification strains *P. variotii* AC70 and *P. variotii* ZW70 as contributions to L-lactic acid biorefinery.

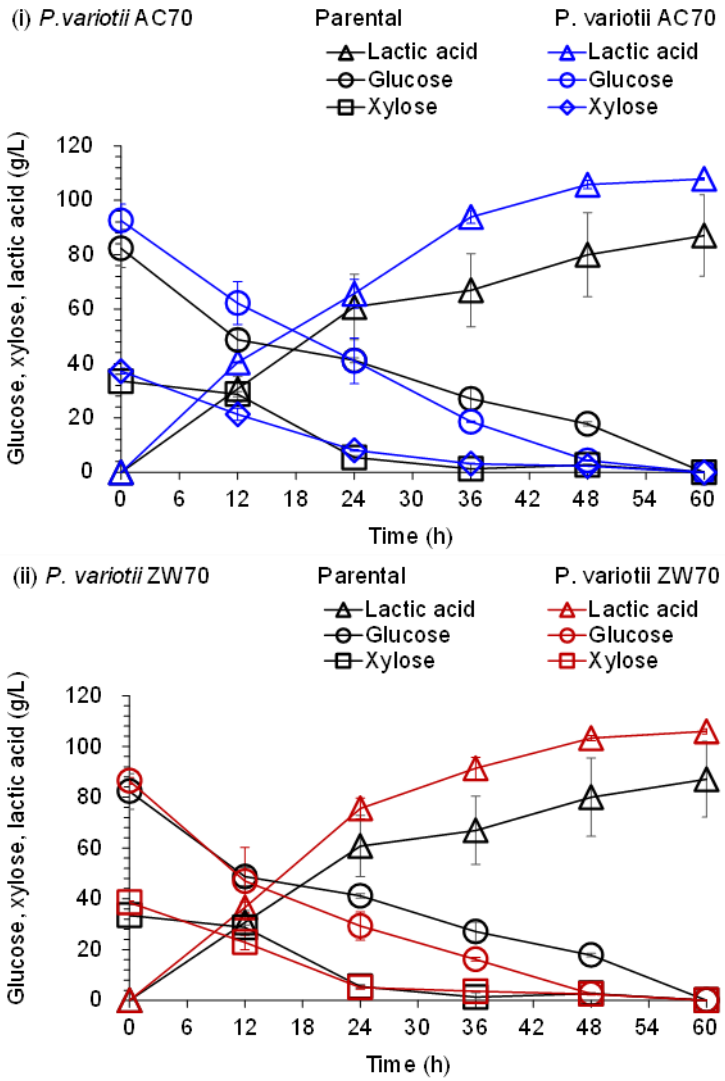


Figure 8 Lactic acid fermentation performance evaluation in the detoxified wheat straw hydrolysate by *P. variotii* AC70 (i) and ZW70 (ii). The fermentation was carried out in a 3 L bioreactor at 42 ◦C with 300 rpm agitation by *P. acidilactici* ZY271 at pH 5.5 by using 25% calcium hydroxide slurry. All data were obtained by two experiments.

2.3.3 Gene transcriptional analysis of the evolved strains

Figure 9 demonstrates the overall scheme of putative assimilation pathways of acetic acid, furfural and HMF in *P. variotii*. Transcriptional regulations of target genes involved in the initial assimilation pathways for acetic acid, furfural, and HMF conversion were analyzed using qRT-PCR, as illustrated in Figure 10.

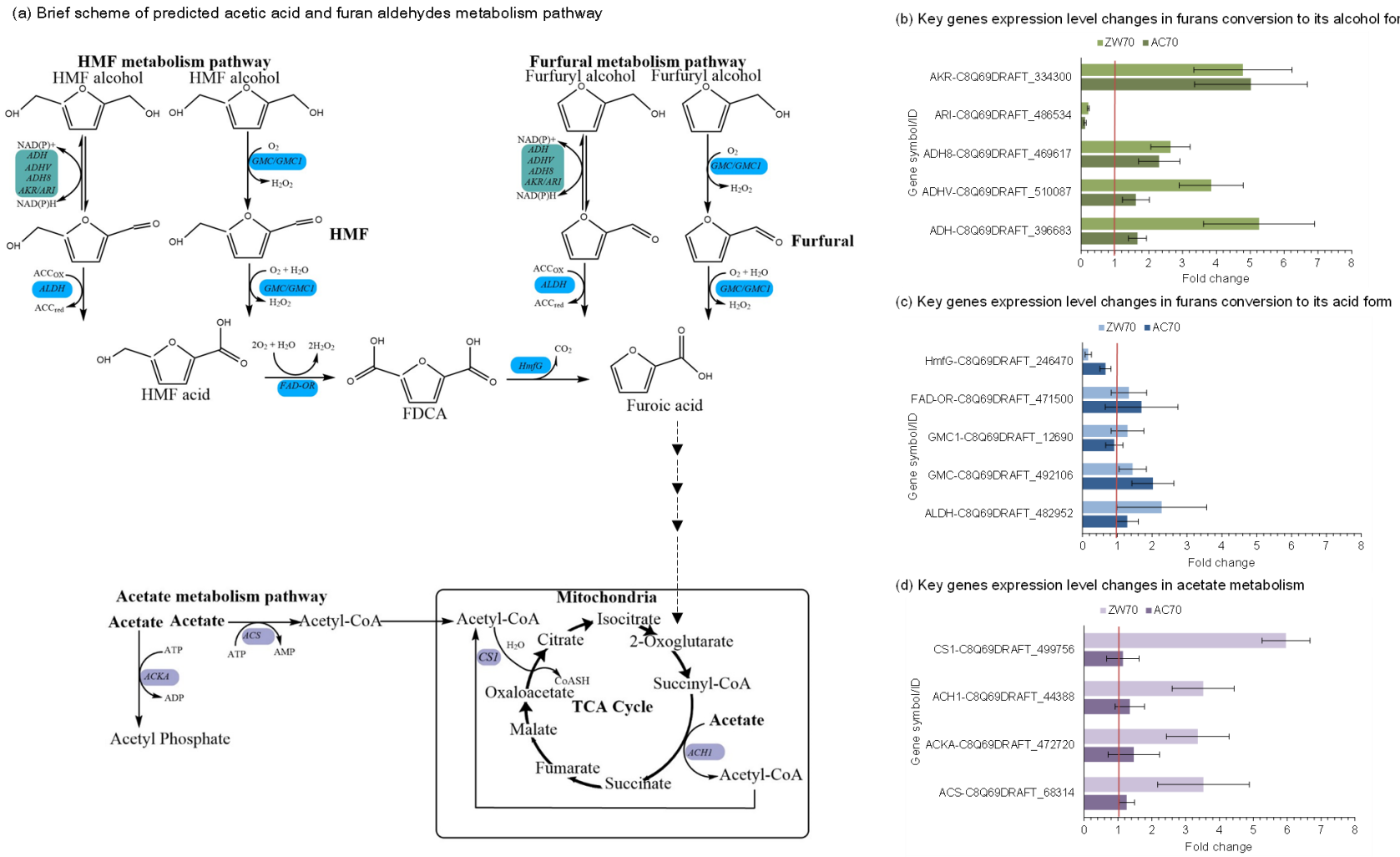


Figure 9 The changes in the expression levels of the key genes related to the metabolic pathways of acetic acid, HMF and furfural in evolved strains compared to parental strain. (a) Initial metabolic pathways. ACS, acetyl coenzyme synthetase, converts acetic acid to acetyl CoA in cytoplasm; ACH1, putative acetyl-CoA hydrolase, converts acetic

acid to acetyl CoA in mitochondria; ACKA, putative acetate kinase: converts acetic acid to acetyl phosphate; CS1, putative citrate synthase; Cit1, catalyzes acetyl-CoA metabolism into citrate. For furan aldehydes metabolism: ADH, alcohol dehydrogenase, catalyzes reversible reduction of furfural/HMF to fufuryl alcohol/HMF alcohol; ADHV, alcohol dehydrogenase V catalyzes reversible reduction of furfural/HMF to furfuryl alcohol/HMF alcohol; ADH8, NADP-dependent alcohol dehydrogenase, catalyzes reduction activity of furfural; ALDH, aldehyde dehydrogenase, oxidizes furfural/HMF to furoic acid/HMF acid; ARI, aldehyde reductase, catalyzes reversible reduction of furfural/HMF to fufuryl alcohol/HMF alcohol; AKR, aldo-keto reductase, catalyzes reversible reduction of furfural/HMF to fufuryl alcohol/HMF alcohol; GMC, putative glucose-methanol-choline oxidoreductase, oxidizes furfural/HMF and furfuryl alcohol/HMF alcohol to furoic acid/HMF acid and; GMC1, putative GMC oxidoreductase, oxidize furfural/HMF and furfuryl alcohol/HMF alcohol to furoic acid/HMF acid; FAD-OR, FAD-dependent oxidoreductase, catalyzes oxidation of HMF acid into FDCA; and HmfG, 3-octaprenyl-4-hydroxybenzoate carboxy-lyase-domain-containing protein, acts as 2,5-furan-dicarboxylic acid decarboxylase 2, catalyzes conversion of FDCA to furoic acid. (b) The changes in the expression levels of the key genes involving in furan aldehydes metabolism into alcohol form. (c) The changes in the expression levels of the key genes involving in furan aldehydes metabolism into furoic acid. (d) 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.

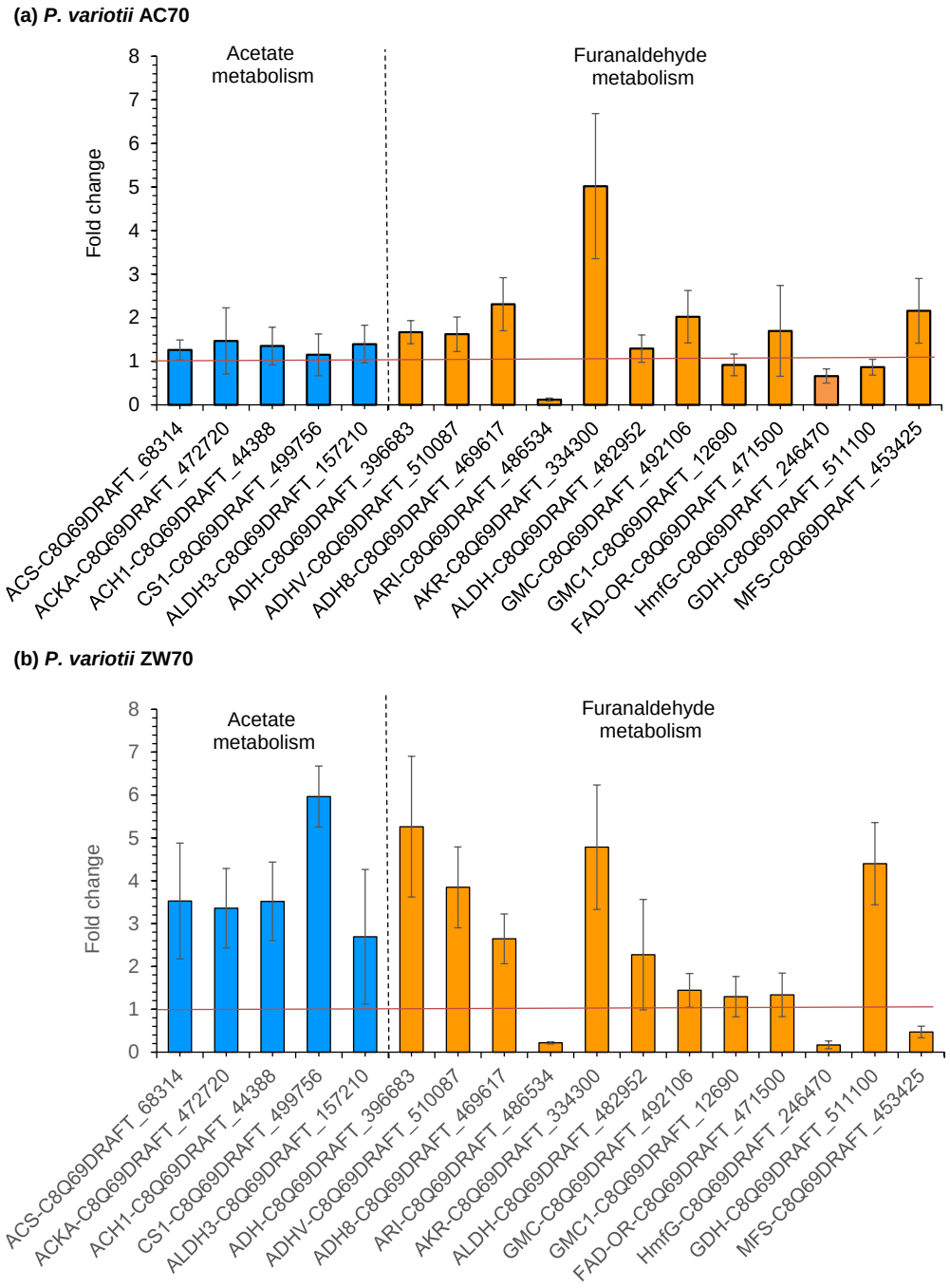


Figure 10 The expression level of potential target genes determined by qRT-PCR. (a) *P. variotii* AC70; (b) *P. variotii* ZW70. Gene expression of progenitor FN89 against all key target genes was assigned as biological control. Error bars represent the standard deviation of the average of three biological replicates.

Acetic acid could be assimilated through three pathways, namely the cytoplasmic formation of acetyl-CoA using acetyl-CoA synthetase (*acs*) with acetyl-AMP as an intermediate, mitochondrial formation of acetyl-CoA through acetyl-CoA hydrolase (*ach1*), and the generation of acetyl phosphate by acetate kinase (*ackA*). In this study, we assayed the transcriptional regulation of the target genes involved in acetate metabolism *acs*, *ach1*, and

ackA in both evolved strains, and the results can be seen in Figure 10. The metabolic response to acetic acid in strains *P. variotii* ZW70 involved significant upregulation of target genes associated with acetic acid assimilation and tolerance. Specifically, the *ACS* gene, encoding acetyl-CoA synthetase, the *ACH* gene, encoding acetyl-CoA hydrolase, and the *ACKA* gene, encoding acetate kinase, were upregulated more than 3-*fold* in evolved strain *P. variotii* ZW70, indicating a concerted effort to metabolize acetic acid. On the other hand, *P. variotii* AC70 showed unclear significant upregulation of these three involved genes. These results explained a better acetic acid degradation improvement of *P. variotii* ZW70 compared with *P.variotii* AC70 and the parental strain in biodetoxification using a synthetic medium supplemented with 15 g/L sodium acetate, 1.5 g/L HMF, and 0.5 g/L furfural. Then, accumulated acetyl-CoA in mitochondria will be further consumed through condensation with oxaloacetate by putative citrate synthase *cit1* to form citrate in mitochondria and enter the TCA cycle for the metabolism process. In addition, genes related to the TCA cycle, such as putative citrate synthase *Cit1* (*CS1*), indicated significant upregulation by more than 5-*fold*, especially in *P. variotii* ZW70. This result suggested its enhanced metabolic activity in acetic acid through assimilation into acetyl CoA and draining into the TCA cycle. *P. variotii* AC70 didn't demonstrate significant differences in *CS1* gene expression level, indicating its consistent results with insignificant upregulation of involved genes in acetyl CoA formation from acetic acid.

The specialization function of aldehyde dehydrogenases ALD3 (*ALDH3*) is involved in β-alanine biosynthesis and involved in the cellular biosynthesis of coenzyme A. Transcriptional level of *ALDH3* gene in evolved *P. variotii* ZW70 demonstrated upregulation more than 2-*fold* which indicated biosynthesis of coenzyme A is more effective to mediate the catalyzation of acetic acid into acetyl-CoA as cofactor in this metabolism pathway. In the evolved *P. variotii* AC70, a noticeable increase in the expression of ALDH3 was not observed. This result indicated that adaptive laboratory evolution in the liquid medium could better induce tolerability towards acetic acid not only by co-upregulation of *acs*, *ach1* and *ackA* genes but also by upregulated *ALDH3* to facilitate efficient metabolism of acetic acid through biosynthesis of coenzyme A.

Notably, the evolved *P. variotii* ZW70 also demonstrates enhancing metabolism to assimilate acetic acid via acetyl phosphate formation mediated by putative acetate kinase, serving as a tolerance mechanism to high acetic acid concentrations. Acetyl-phosphate plays a pivotal role in the tolerance mechanism of acetic acid tolerance by regulating numerous cellular processes in microbes by phosphorylating or acetylating proteins and other molecules, thereby influencing processes ranging from organelle biogenesis to cell cycle regulation[222]. In the *acs* pathway, acetate is catalyzed into acetyl-CoA by *acs* at the expense of ATP and coenzyme A,

yielding adenosine monophosphate (AMP). This process consumes 2 ATP molecules per acetate molecule. Conversely, in the *ackA* pathway, acetate kinase catalyzes acetate into acetyl-phosphate, consuming ATP and producing ADP. The generated ADP can be further converted to ATP via ATP synthetase. Compared to the *acs* pathway, the *ackA* pathway requires less energy to metabolize acetic acid as a carbon substrate to support growth.

Our findings suggest that *P. variotii* ZW70 employs strategies for acetic acid tolerance, utilizing acetyl-CoA generated by acetyl coenzyme synthetase (*ACS*), followed by its incorporation into the TCA cycle via citrate synthase Cit1 (*CS1*). Upregulation of aldehyde dehydrogenase *ald3* in *P. variotii* ZW70 further supports accelerated acetic acid utilization via the *acs* pathway by enhanced biosynthesis of coenzyme A which acts as a cofactor in the metabolism. Moreover, the involvement of acetyl-CoA hydrolase *ach1* in mitochondrial acetate detoxification in *P. variotii* ZW70, particularly, highlights a potential mechanism for mitigating acetic acid toxicity within the mitochondria, emphasizing the complex interplay of metabolic pathways in response to acetic acid stress. Furthermore, *P. variotii* ZW70 also exhibited a tolerance mechanism of acetic acid through its assimilation into acetyl-phosphate formation mediated by the *ACKA* gene. In summary, strain *P. variotii* ZW70 conferred a relatively better detoxification performance in acetic acid degradation based on co-upregulation of *ACS*, *ACH1*, and *ACKA* gene by more than 3-*fold*.

The transcriptional regulation of the target genes involved in conversion pathways of furfural and HMF to furfuryl alcohol and HMF alcohol were catalyzed by alcohol dehydrogenase (*ADH*) and aldo-keto reductase/aldehyde reductase (*AKR*/*ARI*). To gain a deeper insight into the molecular mechanisms involved in furan aldehyde metabolism in evolved *P. variotii* AC70 and *P. variotii* ZW70, we conducted a transcriptional analysis of these target genes by qRT-PCR. As depicted in Figure 10, the results revealed a tolerance response in both evolved strains preceding the upregulation of the *AKR* gene, encoding aldo-keto reductase by more than 4-*fold* and downregulation of *ARI* gene involved in reversible oxidation of HMF and furfural into HMF alcohol and furfuryl alcohol, respectively was observed in both evolved strains. These results suggest a possible explanation that under stress conditions, the co-expression of *AKR* and *ARI* may lead to additional metabolic burden in the cell that causes a further reduction or downregulation of *ARI* in mitigating the metabolic burden in the cell. This co-expression could potentially offset the impact of the *ARI* gene's overexpression on the furfural & HMF degradation activity in evolved strains AC70 and ZW70. In summary, the *AKR* gene exhibits higher activity over *ARI* on furfural and HMF oxidation, participating in the

NAD(P)H-coupled reduction of these compounds to their less toxic alcohol forms in *P. variotii* AC70 and *P. variotii* ZW70.

On the other hand, we carried out transcriptional analysis of three different *ADH* genes, including *ADH* encoding alcohol dehydrogenase, *ADHV* encoding alcohol dehydrogenase class V and *ADH8* encoding NADP-dependent alcohol dehydrogenase which facilitate oxidation of furfural and HMF into its alcohol form. As depicted in Figure 10, we observed a notable upregulation of co-expressed *ADH*, *ADHV* and *ADH8* in evolved *P. variotii* ZW70 by more than 2-*fold*, while *P. variotii* AC70 only showed significant co-expressed *ADH* and *ADH8* by more than 2-*fold*. These results indicated that evolved *P. variotii* AC70 and *P. variotii* ZW70 have preferential reduction activity for HMF and furfural into its less toxic alcohol form, acting as a tolerance response against the inhibitory effects of furan aldehydes. In biodetoxification evaluation in a synthetic medium supplemented with 15 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural, we observed a significant acceleration of furfural degradation of *P. variotii* ZW70 (Figure 7a) that was 12 h shorter conversion efficiency than strain AC70 and progenitor FN89. This condition might be due to the co-upregulation of *ADH*, *ADHV*, *ADH8* and *AKR* that could catalyze the reduction of furfural as well as HMF into its less toxic form. These results explain the higher efficiency of evolved *P. variotii* ZW70 in converting furfural and HMF during the detoxification process in the synthetic medium over *P. variotii* AC70 and parental strain *P. variotii* FN89.

The conversion of furfural and HMF alcohols to furoic acid and HMF acid could be directly oxidized by putative glucose-methanol-choline oxidoreductase (*GMC* and *GMC1*), using molecular oxygen or oxidized by aldehyde dehydrogenases (*ALDH*). Interestingly, we also observed strain *P. variotii* AC70 exhibited upregulation of the *GMC* gene, while strain ZW70 showed upregulation of *ALDH* by more than 2-*fold*. All the target genes on these two pathways were not significantly upregulated in *P. variotii* ZW70 and *P. variotii* AC70 compared to *ADH* and *AKR* genes, indicating the oxidations to acids were the slow conversion compared with the reductions to alcohols. For HMF acid, two further reactions were required to be converted into furoic acid by oxidation into 2,5-furan-dicarboxylic acid (FDCA) through mediation of *FAD-OR* gene encoding FAD-dependent oxidoreductase and then FDCA will be converted to furoic acid catalyzed by gene *HmfG* encoding 3-octaprenyl-4-hydroxybenzoate carboxy-lyase-domain-containing protein. The *FDA-OR* gene showed insignificant upregulation and *HmfG* showed downregulation, 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, and furoyl-CoA synthetase facilitates the oxidation of furoyl-CoA to 5-

hydroxy-2-furoyl-CoA. 5-hydroxy-2-furoyl-CoA is hydrolyzed to 2-oxoglutarate-CoA by presumed spontaneous or catalyzed by an unidentified, nonspecific hydrolase, and then 2-oxoglutarate-CoA will be converted to 2-oxoglutaric acid by enoyl-CoA hydratase/isomerase assigned for 2-oxoglutarate-CoA hydrolase, then enter the central metabolism of TCA cycle.

Glutamate-semialdehyde dehydrogenase (*GDH*) is an enzyme responsible for the in vivo reduction of y-glutamyl phosphate to glutamate 5-semialdehyde, as part of the proline biosynthesis pathway. Interestingly, *P. variotii* ZW70 indicated upregulation of the *GDH* gene more than 4-*fold*, suggesting its enhanced proline biosynthesis as the strategy to improve tolerance and overcome the drawback of lignocellulosic inhibitors during cultivation. These results are similar to *Clostridium acetobutylicum* ATCC 824 which showed its improved tolerance to lignocellulose-derived inhibitors when external proline supplemented in the cultivation media or gene related to proline synthesis was overexpressed[223]. The functions of proline are stabilizing the protein structures, maintaining the cell membrane function, eliminating the intracellular ROS, and lowering the melting temperature of DNA that could serve as an osmolyte, antioxidant, and nutrients or source of energy led to the enhancement response against osmotic stress and oxidative stress[224].

Major facilitator superfamily transporter (*MFS*) is a type of *MDR* (multi-drug resistant) efflux pump found in microbes that involves efflux activity by expelling hydrophobic compounds through the efflux system and is an important mechanism for microbes to resist toxicants and maintain normal cell metabolism. *P. variotii* AC70 demonstrated upregulation of the *MFS* gene by more than 2-*fold*, indicating its other strategies to overcome the inhibition effects of inhibitors by pumping out the toxic compounds from intracellular. On the other hand, *P. variotii* ZW70 showed downregulation of the *MFS* gene. This strategy might involve conferring resistance in cell survival rather than detoxification under an inhibitory environment. Some *MFS* transporters have been overexpressed to enhance microbial tolerance towards toxic compounds. Besides, the furfural/HMF metabolic pathways of *C. basilensis* HMF14 and *P. putida* ALS1267 contain major facilitator superfamily transporters *hmfT2* and *hmfT1*[225, 226].

In conclusion, the observed differential gene expression patterns in evolved *P. variotii* AC70 and *P. variotii* ZW70, particularly in the co-expression of *ADH* and *AKR* genes, contribute to their enhanced efficiency in converting furfural and HMF. Compared with the adaptive laboratory evolution using solid substrate cultivation, the evolved strain obtained from submerged cultivation exhibited a relatively better detoxification performance in a synthetic medium supplemented with an inhibitors cocktail and based on the co-upregulation of target

genes involved in acetic acid and furan aldehydes metabolism by qRT-PCR. This study revealed that adaptive evolution was an effective way to select improved phenotypes under a defined environment for prolonged periods for enhancing the parental biodetoxification fungus *P. variotii*, contributing to an improved tool for the lignocellulose biorefinery process.

2.4 Conclusion

This study demonstrated the adaptive evolution of *P. variotii* under high inhibitor tolerance, using solid-surface and submerged liquid culture methods through a series of stages with gradually increasing inhibitor concentrations, which could boost the tolerance of parental *P. variotii* FN89. Evaluation of biodetoxification revealed enhanced inhibitor conversion rates and cell growth in both evolved strains. Both evolved strains demonstrated a remarkable enhancement in lactic acid fermentation of detoxified hydrolysate, indicating a significant improvement in the overall process. Transcriptional analysis revealed upregulation of target genes involved with acetic acid assimilation and furfural/HMF conversion pathways, particularly in *P. variotii* ZW70, indicating enhanced metabolic activity. The study underscores the effectiveness of adaptive evolution to improve desired phenotypes for enhancing biorefinery processes.

Chapter 3 Isolation of biodetoxification microbes from the high acetic acid hydrolysates of corncob residues

3.1 Background

As a byproduct of furfural production, corncob residue primarily comprises lignin (40.23%), cellulose (38.06%), humic acid (12.35%), trace elements (2.28%), acetic acid (3.55%) and sulfate ions (1.35%). Most of the hemicellulose from agricultural waste (e.g., corn cobs, straws and rice husks) is drained for the furfural production process, leaving behind this residue[227]. Its high salinity and strong acidity pose challenges for further utilization in value-added bioproduct production. Approximately 70% of global furfural production occurs in China, generating 10 to 15 tons of corncob residue per ton of furfural product[228, 229]. Disposal methods like burial or direct burning contribute to air, soil and river pollution, necessitating solutions from furfural enterprises. With cellulose still present in corncob residue, it could serve as a low-cost resource for biorefineries to produce value-added bioproducts.

During furfural production, depolymerization of hemicellulose yields xylose as the main compound, accompanied by fractions of arabinose, mannose, galactose, and glucose. Acetic acid, a common fermentation inhibitor, is released along with other by-products, such as formic acid, levulinic acid, and furan aldehydes (5-hydroxymethylfurfural (HMF) and furfural)[17]. These inhibitors can penetrate cells, altering cytosolic pH, inhibiting growth, and disrupting cellular activities[230, 231]. Detoxification steps are necessary to prepare the hydrolysate for fermentation, typically involving physical, chemical, or biochemical methods, which add complexity and generate waste. In contrast, biodetoxification offers a more environmentally friendly solution, minimizing waste and integrating seamlessly into fermentation. Despite studies on corncob residue utilization for bio-ethanol and bio-butanol production using detoxification approaches[221, 232, 233], biological detoxification of corncob residue hydrolysate remains unexplored.

The corncob residues provided by domestic enterprise contained 31.3 ± 1.4 mg/g of acetic acid and 5.5 ± 0.5 mg/g of HMF. Few studies tried to bioconversion of furfural residues to bioproducts, largely owing to the concentrated inhibitors contained therein. Since the corncob residue possesses a high inhibitor concentration that limits its application for biorefinery and less biological detoxification of this biomass was explored, thus we attempted to isolate and screen microbes present in this biomass as a potential detoxification strain. In this study, we isolated, identified, and characterized microbes from corncob residue to detoxify its hydrolysate.

Enrichment from corncob residue yielded isolates with the potential for removing inhibitors in corncob residue hydrolysate. After screening, we identified *P. variotii* strain CC004, a ubiquitous thermo-tolerant fungus, as a promising candidate for biodetoxification strain for corncob residue. Its detoxification performance was assessed and compared with *P. variotii* FN89, highlighting its potential for enhancing bioprocess efficiency and sustainability for corncob residue. The transcriptional level of target genes involved in acetic acid and HMF metabolism was analyzed by qRT-PCR to investigate the underlying mechanism of its tolerance against corncob residue-derived inhibitors.

3.2 Materials and methods

3.2.1 Raw materials

Corncob residue was selected as the lignocellulose feedstock in this study for microbial isolation and L-lactic acid fermentation, collected from Dalian City, Dalian Province, China, in the fall of 2021. The corncob residues were byproducts from the furfural production. The corncob residue had a moisture content of 53.27%. Subsequently, the composition of corncob residue was analyzed by NREL protocols, revealing 47.47% cellulose, 5.47 ± 0.46 mg/g HMF, and 31.26 ± 1.39 mg/g acetic acid, all reported on a dry weight basis (w/w).

3.2.2 Enzyme and reagents

In this study, the commercial cellulase enzyme Cellic CTec 2.0 was used for the saccharification process, procured from Novozymes, China. The enzyme specifications for filter paper activity, cellobiase activity, and protein content were quantified at 203 FPU/ml, 4900 CBU/ml, and 87.3 mg/ml, respectively, following established protocols (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 purchased from Shanghai Titan Technology Co., Ltd.

3.2.3 Strain and culture

Biological detoxification strain *P. variotii* FN89 (CGMCC 17665) served as the parental strain for evaluation of the detoxification performance. For seed culture preparation, the frozen stock of *P. variotii* FN89 was cultured on a PDA agar plate at 37 °C for 3-4 d to produce spores. Spores were collected by washing the plate with 10 mL of 0.05% (w/v) Tween 80 solution. Subsequently, the spores were gently scraped using a sterilized cell spreader to obtain a spore

suspension. This suspension was then transferred into a 100 mL flask containing seed culture medium (synthetic medium with 20 g/L glucose) and incubated at 37 °C with 300 rpm agitation for 24 h.

The L-lactic acid-producing strain *P. acidilactici* ZY271 (CGMCC 13611, China General Microbiological Culture Collection, Beijing, China) was used for lactic acid fermentation. The seed culture medium comprised Man-Rogosa-Sharp (MRS) medium with 20 g/L glucose, 10 g/L tryptone, 10 g/L Yeast Extract, 5 g/L sodium acetate, 2 g/L ammonium citrate dibasic, 2.6 g/L $K_2HPO_4{\cdot}3H_2O$, 0.58 g/L $MgSO_4{\cdot}7H_2O$ and 0.25 g/L $MnSO_4{\cdot}H_2O$. Activation of frozen stock *P. acidilactici* ZY271 involved inoculation into 20 mL MRS medium in a 100 mL flask and cultivation at 42 °C with 150 rpm agitation for 12 h. Subsequently, seed culture preparation was carried out by transferring 10 mL of the broth into a 500 mL flask containing 100 mL of MRS medium supplemented with glucoamylase at a 1% (w/w) mass ratio to prevent cell flocculation by breaking polysaccharide links among cell aggregations during cultivation. The seed culture was cultivated at 42 °C with 150 rpm agitation for 8 h.

3.2.4 Isolation of microorganism

The corncob residue sample's pH was adjusted to 5.5 using 25% calcium hydroxide. Subsequently, the adjusted sample was exposed to ambient air for one month, maintaining moisture by periodic water spraying. The sample was left standing until contamination became visibly apparent on the sample. Following contamination, the sample underwent homogeneous mixing, after which 1 g of the contaminated sample was transferred into a tube containing 9 mL of sterile saline solution (0.9% w/v NaCl) and mixed thoroughly. Subsequently, 100 μL of the resulting suspensions were mixed with 900 μL of sterilized saline solution iteratively until a dilution factor of 10^6 was achieved. Portions of the suspension with dilution ratios of 10^5 and 10^6 were then spread onto PDA and LB agar medium and incubated at 30 °C and 37 °C for 48 h to facilitate microbial population observation. Single colonies exhibiting varying morphologies were selected and consecutively re-streaked onto PDA agar plates until the strains were purified.

For the screening, the isolates were grown in PDA agar medium with enrichment of

multiple inhibitors as below at 37 °C for 1–5 days:

1st stage screening: 5 g/L sodium acetate + 1.5 g/L HMF + 1.5 g/L furfural

2nd stage screening: 9 g/L sodium acetate + 1.5 g/L HMF + 0.5 g/L furfural

3rd stage screening: 11 g/L sodium acetate + 1.5 g/L HMF + 0.5 g/L furfural

4th stage screening: 15 g/L sodium acetate + 1.5 g/L HMF + 0.5 g/L furfural

3.2.5 Molecular identification

3.2.5.1 DNA extraction

Each strain was cultured on PDA agar plates at a constant temperature of 37 °C to harvest the cell mass for DNA extraction. Subsequently, the cell mass was collected and transferred into sterilized 2 mL Eppendorf tubes. The cell mass was ground using liquid nitrogen to achieve fine powder. Afterward, the ground cell mass was resuspended and lysed in 700 μL of lysis buffer. The lysis buffer composition included Tris-HCl (2.42 g), sodium acetate (1.36 g), ethylenediaminetetraacetic acid (EDTA) (1.86 g), and sodium dodecyl sulfate (SDS) (5 g), diluted in double distilled water with pH adjusted to 8.0. The mixture was then vortexed and incubated at room temperature for 10 minutes. Next, 700 μL of DNA extraction phenol solution (from Solarbio Life Sciences, China) was added to the tube, followed by thorough mixing. After centrifugation at 12000 rpm at 4 °C for 10 min, 400 μL of the supernatant was promptly transferred to a fresh tube, followed by the addition of 400 μL of trichloromethane. The resulting solution was then gently mixed until it turned milky and then centrifuged at 12000 rpm at 4 °C for 10 minutes. Subsequently, 350 μL of the supernatant was transferred to a fresh tube, followed by the addition of 245 μL isopropanol, and then incubated at -20 °C for 30 min. Following another centrifugation step under the same conditions, the aqueous phase was removed, and 500 μL of 75% ethanol was added into the tube to wash the precipitated DNA. Afterward, the DNA was dried, dissolved in 50 μL of double distilled water, and vortexed. Subsequently, the integrity and quantity of the total DNA were analyzed based on the OD_{260}/OD_{280} ratio using a DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Finally, the total DNA was stored in a -20 °C freezer for subsequent molecular identification.

3.2.5.2 18S rDNA-ITS sequencing

The DNA extracted from each strain underwent characterization through 18S rDNA-ITS gene sequencing. The 18S rDNA gene was sequenced using universal primers ITS 1 and ITS 4, with the respective sequences 5′- TCCGTAGGTGAACCTGCGG -3′ and 5′- TCCTCCGCTTATTGATATGC 3′. The cycling conditions used for conducting PCR were as follows: initial denaturation at 95 °C for 5 min; denaturation at 95 °C for 30 s, followed by 35 cycles of primer binding at 61 °C for 15 s and elongation at 72 °C for 30 s; concluding with an end extension at 72 ◦C for 10 min and a 16 ◦C soak. Afterward, the amplified products were sequenced by Sangon Biotech (Shanghai, China). Identification of sequences exhibiting similarity (> 97%) to the 18S rDNA-ITS gene of the isolated strains was analyzed using BLAST searches.

3.2.6 Corncob residue hydrolysate preparation

In a 5 L bioreactor, the corncob residue was subjected to hydrolysis through a fed-batch process within 24 h, using a helical ribbon impeller at 50 °C. In addition, the cellulase enzyme Cellic CTec 2.0 was added into the bioreactor at 4 mg of cellulase proteins per gram of dry corncob residue. The final solid loading was 25% (w/w), and the pH of corncob residue was adjusted to approximately 5.5 using 25% calcium hydroxide before loading to the bioreactor. Celtic CTec 2.0 cellulase enzyme was added to the bioreactor when the temperature reached 50 °C to maintain enzyme activity. The hydrolysis process was carried out with an agitation speed of 200 rpm and at a temperature of 50 °C for 24 h. The inhibitors composition of the 25% (w/w) corncob residue hydrolysate consist of 13.42 – 14.71 g/L acetic acid, 2.33 – 2.49 g/L HMF, and 2.50 – 2.80 g/L formic acid.

3.2.7 Detoxification assays

3.2.7.1 Pre-evaluation of detoxification assays

Hydrolysis of corncob residue was performed in shaking flasks for 24 h under 150 rpm agitation at 50 °C with a final solid loading of 20% (w/w). The initial pH was adjusted to 5.5 using calcium hydroxide. Celtic Ctec 2.0 cellulase enzyme was added to the solution with a

concentration of 4 mg protein/g dry matter (DM).

Spore suspensions of four new isolates were initially collected by washing PDA agar plates with 10 mL of 0.05% (w/v) Tween 80 solution. The seed culture was prepared by transferring the spore suspension into a synthetic medium containing 20 g/L glucose and cultivated for 24 h under 300 rpm agitation at 37 °C. Pre-evaluation of detoxification assays was conducted in 250 mL shake flasks containing 50 mL of 20% (w/w) corncob residue hydrolysate for evaluating detoxification performance among four isolates. Detoxification was initiated by inoculating a 10% seed culture of each newly isolated strain into the hydrolysate under 300 rpm agitation at 37 °C for 48 h. Periodic samples were collected to analyze inhibitor and sugar residual concentrations during detoxification.

The two best isolates underwent another evaluation round for detoxification performance comparison with *P. variotii* FN89 in shaking flasks. The evaluation was performed in 250 mL shaking flasks containing 50 mL of 20% (w/w) corncob residue hydrolysate to compare the detoxification performance between the best isolates and *P. variotii* FN89. Detoxification was initiated by inoculating a 10% seed culture into the hydrolysate under 300 rpm agitation at 37 °C for 48 h. Periodic samples were collected to analyze inhibitor and sugar residual concentrations during detoxification.

3.2.7.2 Comparison of detoxification performance of *P. variotii* CC004 and *P. variotii* FN89

Detoxification assays were performed in a 3 L bioreactor using undetoxified 25% (w/w) wheat straw hydrolysate. The detoxification was carried out by inoculating a 10% seed culture of *P. variotii* FN89 and *P. variotii* CC004. Detoxification conditions for corncob residue hydrolysate included 500 rpm agitation at 37 °C and 0.5 vvm aeration. Periodic samples were collected to analyze inhibitor and sugar residual concentrations during detoxification.

3.2.8 Cellulosic L-lactic acid fermentation performance evaluation

Detoxified corncob residue hydrolysate by each strain underwent lactic acid fermentation directly in a 3 L bioreactor. The fermentation process was initiated by inoculating 10% of *P. acidilactici* ZY271's seed culture into detoxified corncob residue hydrolysate and adding nutritional salts (15 g/L YE, 10 g/L tryptone, 2 g/L ammonium citrate dibasic and 0.25 g/L

$MnSO_4 \cdot H_2O$). Fermentation occurred in anaerobic conditions at 42 °C with 300 rpm agitation for 72 h without aeration. The pH during fermentation was maintained at 5.5 using 25% calcium hydroxide. Periodic samples were collected to analyze lactic acid and sugar residual concentrations during fermentation.

3.2.9 RNA extraction and qRT-PCR analysis for selected genes

The procedures of RNA extraction and qRT-PCR analysis for acetic acid and furan aldehyde metabolism involved target genes were the same as in Chapter 2, 2.2.9.

3.2.10 Analytical methods

Analysis of glucose, xylose, lactic acid, acetic acid, 5-hydroxymethylfurfural (HMF), and furfural was determined using the same procedures as in Chapter 2, 2.2.10.

3.3 Results and Discussion

3.3.1 Isolation, screening, and identification of detoxification strain

In the isolation stage, four distinct isolates with varying phenotypes were obtained from corncob residue samples cultivated on PDA and LB agar plates, designated as CC001, CC002, CC003, and CC004. Subsequently, all four isolates underwent purification and subsequent screening stages.

To identify microorganisms capable of thriving in a toxic environment, the screening involved streaking the four isolates into a screening medium to select tolerant phenotypes. Given that acetic acid, HMF and furfural were the primary inhibitors present in lignocellulose hydrolysate, the ability of microbial growth in the screening medium containing these inhibitors served as a criterion for screening isolates with toxin tolerance phenotypes. The screening stage encompassed the use of five different media to evaluate the capability of all isolates to grow on PDA agar plates supplemented with varying concentrations of sodium acetate, HMF, and furfural. Table 4 outlines the growth performance of the four new isolates on the screening medium. Notably, only isolate CC004 demonstrated cell viability, exhibiting growth in PDA agar plates enriched with 15 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural. In addition, the isolate CC002 displayed the most severe growth inhibition, with no cell growth on agar plates when the sodium acetate concentration increased to 9 g/L. Among all isolates, CC004

exhibited significantly superior growth performance on PDA agar plates enriched with sodium acetate, up to a concentration of 15 g/L, along with 1.5 g/L HMF and 0.5 g/L furfural. Following closely, the isolate CC001 demonstrated the ability to grow on PDA agar plates enriched with sodium acetate up to a concentration of 11 g/L, along with 1.5 g/L HMF and 0.5 g/L furfural. These results indicate that the sensitivity of CC002 to sodium acetate was the highest, followed by CC003 and CC001, respectively.

Table 4 Growth performance of four isolates on PDA agar plate supplemented with different inhibitor concentrations.

Isolates	Screening medium A	Screening medium B	Screening medium C	Screening medium D	Screening medium E
CC001	+++	+++	+++	+++	-
CC002	+++	++	-	-	-
CC003	+++	+	+	-	-
CC004	+++	+++	+++	+++	+++

"-" indicates no colony. "+" indicates cell growth but minimal (< 8 single colonies), ++ indicates medium cell growth (8 – 20 single colonies), and +++ indicates fast cell growth (> 20 single colonies). The dilution factor for screening mediums A & B was 10^5 and the dilution factor for screening mediums C, D, and E was 10^4. Screening medium A contains 5 g/L sodium acetate, 1.5 g/L HMF and 1.5 g/L furfural; screening medium B contains 7 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural; screening medium C contains 9 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural; screening medium D contains 11 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural; and screening medium E contains 15 g/L sodium acetate, 1.5 g/L HMF and 0.5 g/L furfural.

To further characterize the four isolates, their morphology was analyzed by cultivating them in PDA agar plates, as depicted in Figure 11. Colonies of all isolates exhibited dispersed filaments, with a suede-like surface comprising dense conidiophores and a yellowish-green color. Additionally, a powdery surface with irregular edges was observed for all isolates. Notably, each isolate displayed a different dense color in the colony center, with CC004 showing the deepest green color, followed by CC003 and CC002, while CC001 exhibited the lightest color. Moreover, the conidiophores of CC002, CC003, and CC004 appeared denser than CC001.

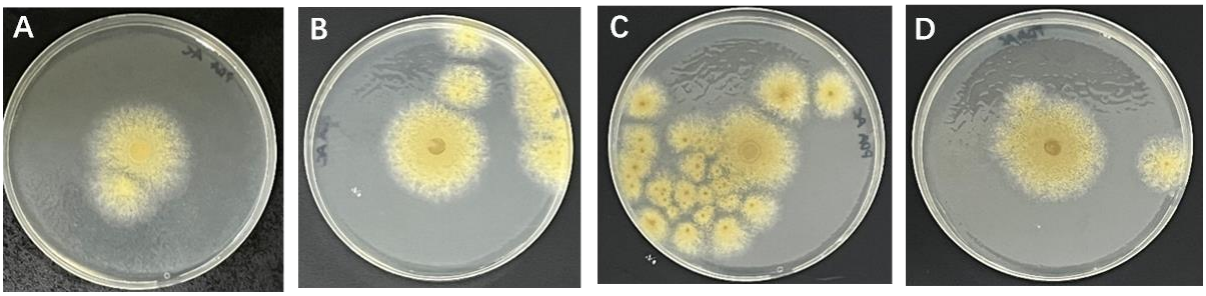


Figure 11 Morphology of the isolates. A. CC001; B. CC002; C. CC003 and D. CC004. All isolates were grown on a PDA agar plate for 48 h at 37 ºC.

The identities of the four isolates were determined through 18S rDNA-ITS sequencing. Analysis of the 18S rDNA-ITS gene revealed a consistent amplification of the ITS1 and ITS4 regions, ranging between 500 and 750 base pairs for all isolates (Figure 12). By utilizing Nucleotide BLAST with the 18S rDNA-ITS sequences, the isolates were identified as the genus *Paecilomyces*, corresponding to the genera *Byssochlamys*, and species *Paecilomyces variotii*. Isolate CC003 and CC004 exhibited a 100% percent identity with *P. variotii* ST017, while CC001 and CC002 showed percent identities of 99.8% and 99%, respectively, with *P. variotii* ST017. The construction of the phylogenetic tree is presented in Figure 13, based on sequencing analysis.

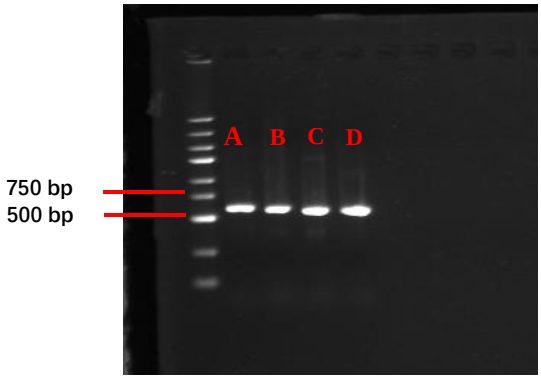


Figure 12 Amplification of ITS 1 and ITS 4 regions from four isolates with the size of the region found to be approximately between 500 bp to 750 bp. A. CC001, B. CC002, C. CC003, D. CC004.

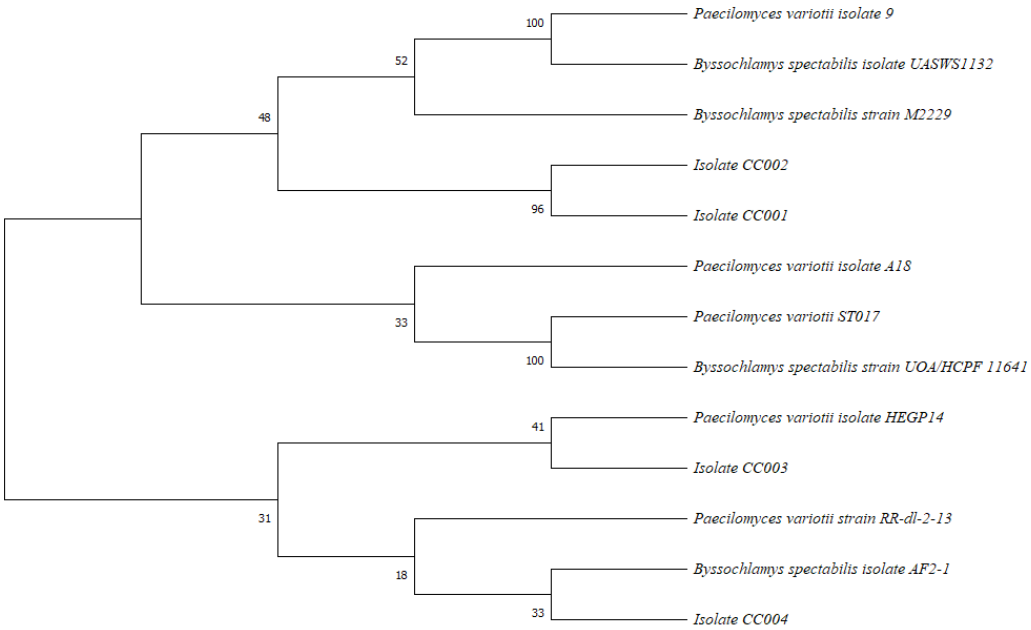


Figure 13 The phylogenetic tree of all isolates based on 18S rDNA-ITS sequences was constructed by MEGA 11 software according to the neighbor-joining method with 1000 bootstrap replicates, describing the phylogeny position of all isolates. The scale bar represents 0.05 nucleotide substitutions per position.

3.3.2 Pre-evaluation assays for detoxification performance

Four isolates were introduced into 20% (w/w) corncob residue hydrolysate to assess their detoxification efficacy in shaking flasks for 48 h at 37 °C, with agitation set at 300 rpm. Subsequently, sugar consumption and inhibitor residuals were quantified as evaluation measurements. As depicted in Figure 14, no significant discrepancies were observed in the formic acid removal capacity among the four isolates, all exhibiting complete degradation of 1.56 ± 0.11 g/L formic acid after 4 h of cultivation. Moreover, all isolates indicated similar behavior in glucose consumption. Isolates CC001 and CC004 exclusively eliminated HMF after 24 h of cultivation from an initial concentration of 1.23 ± 0.01 g/L, while residual HMF levels of 0.01–0.08 g/L persisted in corncob residue hydrolysate cultured with isolate CC002 and CC003 at the 24 h cultivation. For acetic acid depletion, isolate CC002 exhibited 66% removal with a residual concentration of 3.41 ± 1.82 g/L after 48 h cultivation, making it the most sensitive isolate. During the 48 h cultivation period, the strains identified as CC001 and CC004 exhibited exceptional abilities to deplete acetic acid, with 84% and 89% depletion rates, respectively. Moreover, their residual levels remained under 1.7 g/L after the process, indicating their efficiency. The preliminary screening using 20% (w/w) corncob residue hydrolysate consistently mirrored the growth performance results of the isolates on screening media supplemented with varying concentrations of acetic acid, HMF, and furfural in which isolate

CC004 demonstrated the best performance in detoxification efficacy. Overall, CC004 exhibited notable degradation efficacy in acetic acid and HMF, followed by CC001, compared to CC002 and CC003. Based on pre-evaluation results, we selected CC001 and CC004 for further analysis and characterization.

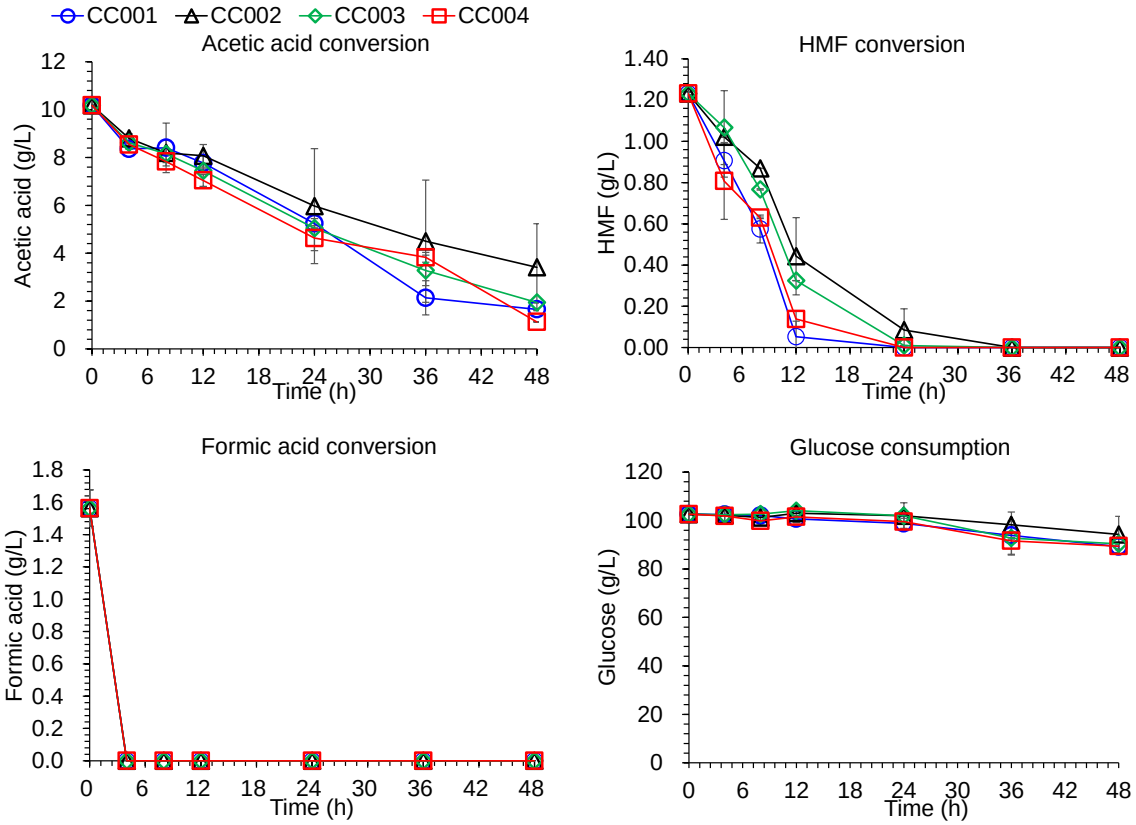


Figure 14 Detoxification pre-evaluation of four isolates in 20% w/w corncob residue hydrolysate. The detoxification was carried out in a shaking flask at 37 ºC with 300 rpm agitation for 48 h. All data were obtained by two experiments and presented as mean ± SD.

Further pre-evaluation was executed by comparing detoxification performance between the best isolates CC001 and CC004 with *P. variotii* FN89 in shaking flasks using 20% (w/w) corncob residue hydrolysate. As depicted in Figure 15, unclear differences were observed among all strains in glucose consumption, indicating consistent results that the new isolates performed similar behavior in glucose utilization to *P. variotii* FN89 during the 48 h detoxification process. Formic acid degradation was consistent with the previous experiment, demonstrating complete degradation after 4 h of cultivation by isolates CC001 and CC004. Interestingly, *P. variotii* FN89 showed its capability to degrade formic acid within 4 h. However, for HMF depletion, significant differences were observed in which isolate CC004 indicated almost complete depletion of HMF, with a residual of 0.05 g/L after 36 h of cultivation, showing

a 27.43% better efficiency than *P. variotii* FN89. While isolate CC001 exhibited a slower degradation rate at 36 h compared to *P. variotii* FN89, with a residual of 0.74 g/L HMF, which was 0.22 g/L less efficient than *P. variotii* FN89. After 48 h of cultivation, isolate CC001 and *P. variotii* FN89 demonstrated a similar degradation rate of HMF. For acetic acid depletion, we observed a significantly enhanced degradation rate on isolate CC004, which achieved complete degradation after 48 h of cultivation, while 2.28 ± 1.97 g/L and 2.02 ± 0.01 g/L of acetic acid residual were still detected on isolates CC001 and strain FN89, respectively. In addition, we observed that *P. variotii* FN89 and both isolates CC001 and CC004 preferentially depleted formic acid over HMF and acetic acid. Faster degradation of acetic acid was observed on isolate CC004 after achieving almost complete degradation of HMF, indicating prior degradation of furan aldehyde over acetic acid. While isolate CC001 and strain FN89 did not achieve complete degradation of HMF at 36 h, indicating inhibition in acetic acid metabolism. These results showed the inhibition of acetic acid utilization as a carbon source by *P. variotii* when furan aldehyde existed in the solution. As isolate CC004 outperformed against isolate CC001, isolate CC004 was used for further characterization in detoxification evaluation.

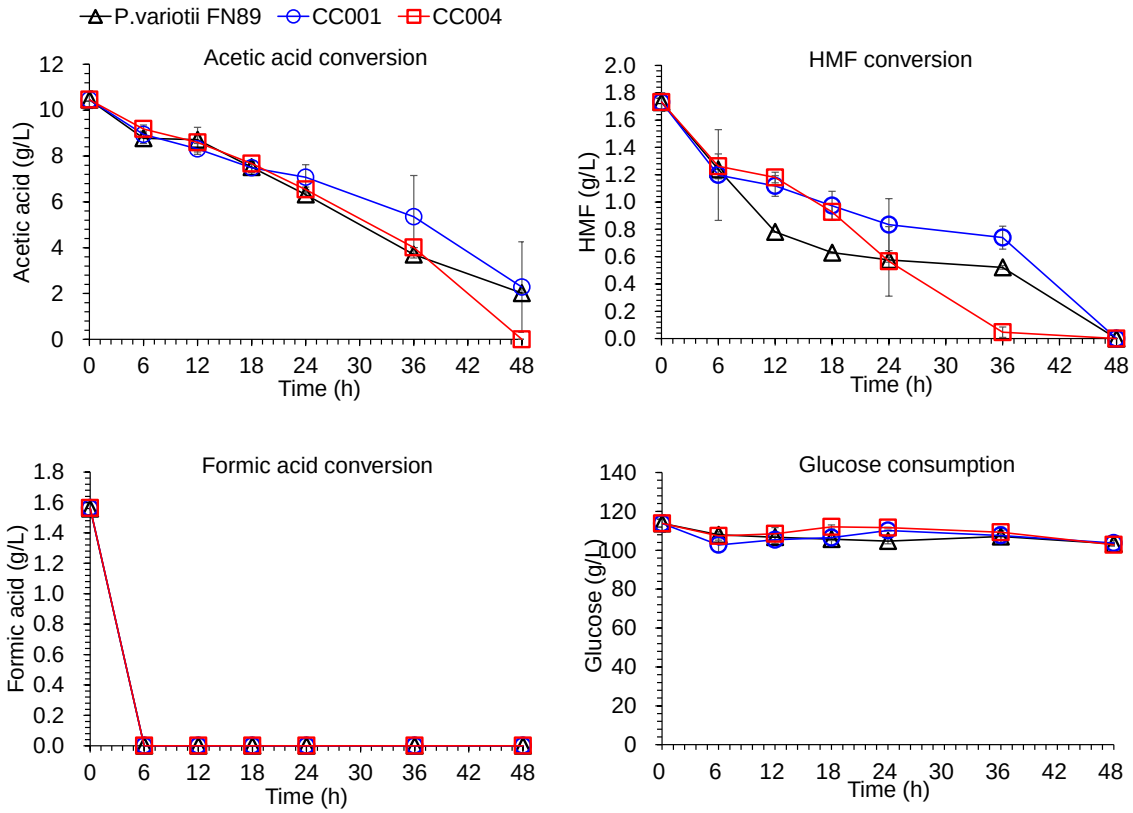


Figure 15 Detoxification evaluation between the isolate CC001 and CC004 with *P. variotii* FN89 in 20% w/w corncob residue hydrolysate. The detoxification was carried out in a shaking flask at 37 ◦C with 300 rpm agitation for 48 h. All data were obtained by two experiments and presented as mean ± SD.

3.3.3 Detoxification performance for two isolates and subsequent cellulosic L-lactic acid fermentation

To simulate the industrial application of biological detoxification of corncob residue hydrolysate and utilize the bio-detoxified hydrolysate for cellulosic L-lactic acid production, we conducted biological detoxification and L-lactic acid fermentation in a 3 L bioreactor. The detoxification performance of the superior isolate, CC004, was compared to that of *P. variotii* FN89 under higher solid loading conditions (25% w/w) of corncob residue. The results presented in Figure 16 revealed similar glucose consumption and formic acid depletion during the detoxification process, indicating consistency with previous experiments in shaking flasks. Notably, isolate CC004 exhibited a 40.39% higher degradation of HMF compared to *P. variotii* FN89 after 24 h of cultivation. Almost complete degradation of HMF was achieved within 24 h for isolate CC004, with a residual HMF concentration of 0.09 g/L. By contrast, *P. variotii* FN89 required 34 h to eliminate total HMF presence in the hydrolysate, suggesting a 10 h longer duration than isolate CC004. In addition, isolate CC004 demonstrated superior efficiency in acetic acid degradation with a residual of 3.18 g/L after 30 h cultivation and achieved almost complete degradation after 34 h of cultivation. At 30 h, *P. variotii* FN89 still exhibited a residual acetic acid concentration of 6.52 g/L, representing a 25.63% lower reduction rate than isolate CC004. Overall, isolate CC004 showcased remarkable detoxification performance over *P. variotii* FN89 in corncob residue hydrolysate. Under optimum conditions of aeration and agitation in the bioreactor, the degradation rate was more efficient than that observed in shaking flasks. Preferential HMF and acetic acid utilization and negligible sugar consumption during the detoxification process by *P. variotii* CC004 offer its advantage as a potential detoxification strain in cellulosic bioproduct refinery.

Previous studies on the biological detoxification of corncob residue were scarce, and traditional detoxification is cost-ineffective and not eco-friendly, which has hindered its favorability as a lignocellulosic material for microbial fermentation. Previous studies on utilizing corncob residue for butanol production involved detoxification methods, such as water rinsing, resin, and activated carbon[232]. However, water rinsing, aimed at removing acids and inhibitors, risks sugar loss and generates wastewater containing toxic compounds, thereby incurring additional wastewater treatment costs. Moreover, water rinsing leads to lower solid loading. Resin detoxification is cost-ineffective and lacks feasibility for industrial purposes, while activated carbon detoxification, although low-cost, results in the loss of fermentable sugars. In contrast, this study introduces a cost-effective and environmentally friendly

detoxification method utilizing the tolerant strain *P. variotii* CC004. This biodetoxification strain effectively removes formic acid and HMF before acetic acid, with only a low concentration of acetic acid (less than 3.2 g/L) remaining in the hydrolysate after 30 h cultivation, which can be tolerated by fermentative strains, thereby saving detoxification time.

Biodetoxification was terminated after 34 h cultivation, and each bio-detoxified hydrolysate was subjected to L-lactic acid fermentation by *P. acidilactici* ZY271 to assess the detoxification effectivity by isolate CC004 and *P. variotii* FN89 for the subsequent fermentation process. As depicted in Figure 17, the detoxified corncob residue hydrolysate inoculated by isolate CC004 consistently exhibited a higher accumulation of lactic acid, displaying a 12.18% increase compared to the hydrolysate detoxified by *P. variotii* FN89. The lactic acid productivity of detoxified hydrolysate by isolate CC004 was 1.45 g/Lh^{-1}, indicating a 12.18% improvement compared with detoxified hydrolysate by strain FN89. This difference could be attributed to the higher residual acetic acid level (4.68 g/L) in the detoxified hydrolysate by *P. variotii* FN89, which decreased the fermentation productivity of *P. acidilactici* ZY271. Furthermore, the sugar utilization rate of *P. acidilactici* ZY271 in the detoxified hydrolysate by *P. variotii* FN89 was significantly slower after 12 h of cultivation. In contrast, almost complete sugar utilization was achieved within 60 h of cultivation for the detoxified hydrolysate by isolate CC004, representing a 12 h shorter fermentation period compared to *P. variotii* FN89. This result suggests that higher initial concentrations of acetic acid can impede the conversion rate of fermentable sugars to L-lactic acid by *P. acidilactici* ZY271.

Previous studies on utilizing corncob residue as a substrate for microbial fermentation, particularly for lactic acid production, were less reported. Prior research predominantly focused on employing lower solid loading of corncob residue combined with other carbohydrate sources like corn saccharification liquid (CR) and yeast hydrolysate (YH). The study reported a lactic acid accumulation of 40.74 g/L after 120 h of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* cultivation as lactic acid-producing strains [218]. However, this approach may incur additional operational costs in industrial L-lactic acid biorefinery applications. In contrast, our study demonstrated enhanced L-lactic acid fermentation by utilizing a high solid loading of corncob residue at 25% (w/w) without additional carbohydrate sources. These results showed a significant improvement, with L-lactic acid reaching 104.09 ± 0.31 g/L and complete glucose utilization within 48 h of *P. acidilactici* ZY271 cultivation. Notably, this achievement represents a 12.18% increase in lactic acid production and offers a fermentation time efficiency of 72 h.

In summary, isolate CC004 exhibited a better degradation rate of acetic acid and HMF presence in 25% (w/w) corncob residue hydrolysate compared to *P. variotii* FN89, which was

consistent with previous pre-evaluation in shaking flask but under optimum condition aeration and agitation in 3 L bioreactor, the degradation rate was more efficiency than those in shaking flask. These results suggested that isolate CC004 could be employed as a potential detoxification strain to remove inhibitors from corncob residue hydrolysate for the efficient lactic acid fermentation of *P. acidilactici* ZY271. This study provides the newly isolated detoxification strain *P. variotii* strain CC004 and its application as a contribution to lactic acid biorefinery by using corncob residue.

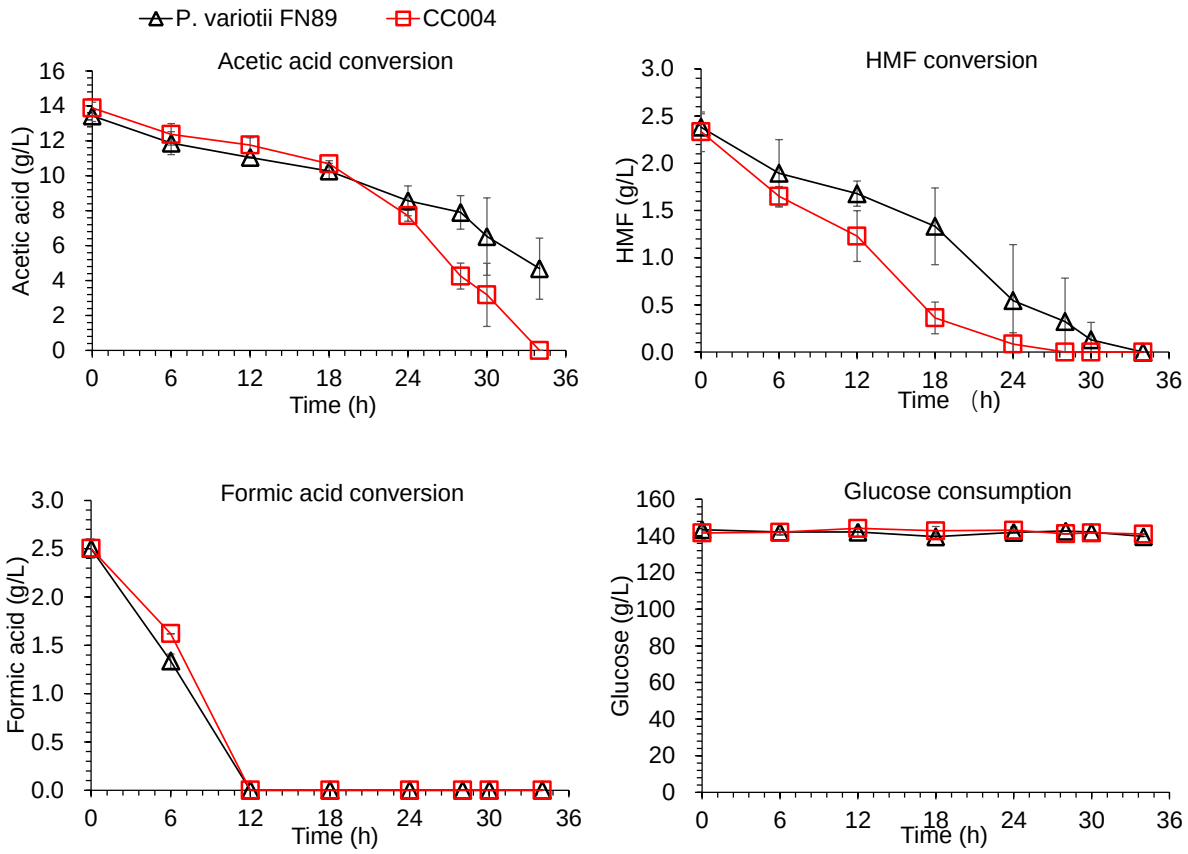


Figure 16 Biodetoxification evaluation in high inhibitors containing corncob residue hydrolysate. The detoxification was carried out in a 3L bioreactor at 37 ºC with 500 rpm agitation and 0.5 vvm aeration. All data were obtained by two experiments and presented as mean ± SD.

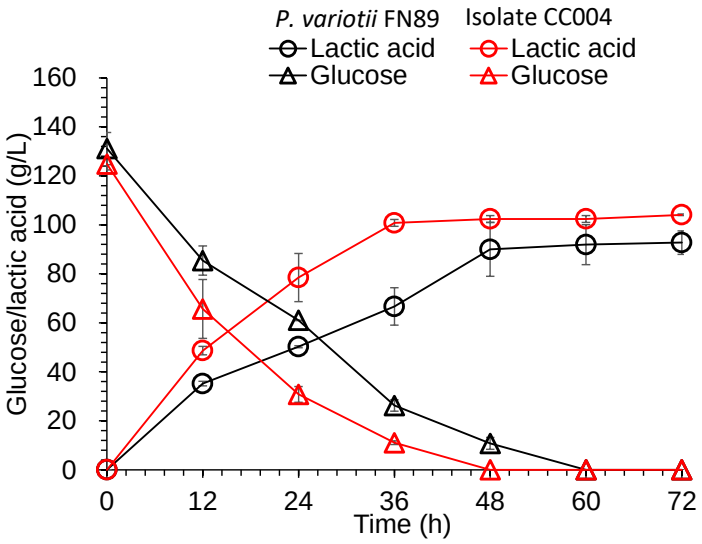


Figure 17 Lactic acid fermentation performance evaluation in the detoxified corncob residue hydrolysate by *P. variotii* FN89 and isolate CC004. The fermentation was carried out in a 3L bioreactor at 42 ºC with 300 rpm agitation by *P. acidilactici* ZY271 at pH 5.5 by using 25% calcium hydroxide slurry. All data were obtained by two experiments.

3.3.4 Gene transcriptional level of the new isolate

A previous study revealed that tolerant strains demonstrated higher expression levels of target genes involved in acetic acid and furan aldehydes metabolism. To investigate the molecular mechanism of acetic acid tolerance of isolate CC004, we performed transcriptional analysis to evaluate the expression level of target genes related to acetic acid metabolism, including *acs* gene encoding acetyl-CoA synthetase, *ackA* encoding acetate kinase and *ach1* encoding acetate hydrolase. As depicted in Figure 18, significant co-upregulation of gene *ACS* and *ACH1* was observed in isolate CC004, with expression levels of both genes being 3-*fold* higher than that of strain FN89, suggesting that isolate CC004 exhibits a preference for assimilating acetic acid into acetyl-CoA over acetyl-phosphate showing by significant downregulation of *ACKA* gene. Both *ACS* and *ACH1* genes mediate the assimilation of acetic acid through the formation of acetyl-CoA in cytoplasm and mitochondria, respectively, indicating mechanism tolerance of acetic acid in isolate CC004 by synthesizing acetyl-CoA from acetic acid.

The accumulation of acetyl-CoA in the cell is then directed toward the glyoxylate shunt (via isocitrate lyase and malate synthase outside the mitochondria) or enters the mitochondria to be metabolized in the TCA cycle. Given that isolate CC004 primarily assimilates acetic acid into acetyl-CoA, we hypothesized that subsequent pathways in acetyl-CoA metabolism leading into the TCA cycle might be upregulated. To verify this assumption, we conducted

transcriptional analysis of the *CS1* gene, which encodes citrate synthase *cit1*, a crucial enzyme facilitating the utilization of acetyl-CoA through condensation with oxaloacetate to form citrate in the mitochondria, subsequently integrating it into the TCA cycle for further metabolic processing. As depicted in **Figure 18**, we observed significant upregulation of the *CS1* gene, confirming the accumulation of acetyl-CoA from acetic acid and the induction of subsequent metabolism of acetyl-CoA into citrate, thus entering the TCA cycle.

Assimilation of acetic acid into acetyl-CoA requires utilization of ATP and CoA (coenzyme A) and form monophosphate (acetyl-AMP) as an intermediate during acetyl-CoA catalyzed conversion by *ACS*. Aldehyde dehydrogenases *ALD3* (*ALDH3*) plays a role in β-alanine biosynthesis and is involved in the cellular biosynthesis of coenzyme A. Given that the *ACS* gene was less preferred than the *ACH1* gene, we assumed that the demand of coenzyme A in the metabolism pathway might not highly trigger the expression level of *ALDH3* for the biosynthesis of coenzyme A. To verify this assumption, we performed transcriptional analysis for the *ALDH3* gene expression level. The result verified our assumption by indicating significant downregulation of the *ALDH3* expression level, confirming a higher expression level of *ACH1* over *ACS* in assimilating acetic acid into acetyl-CoA without coenzyme A.

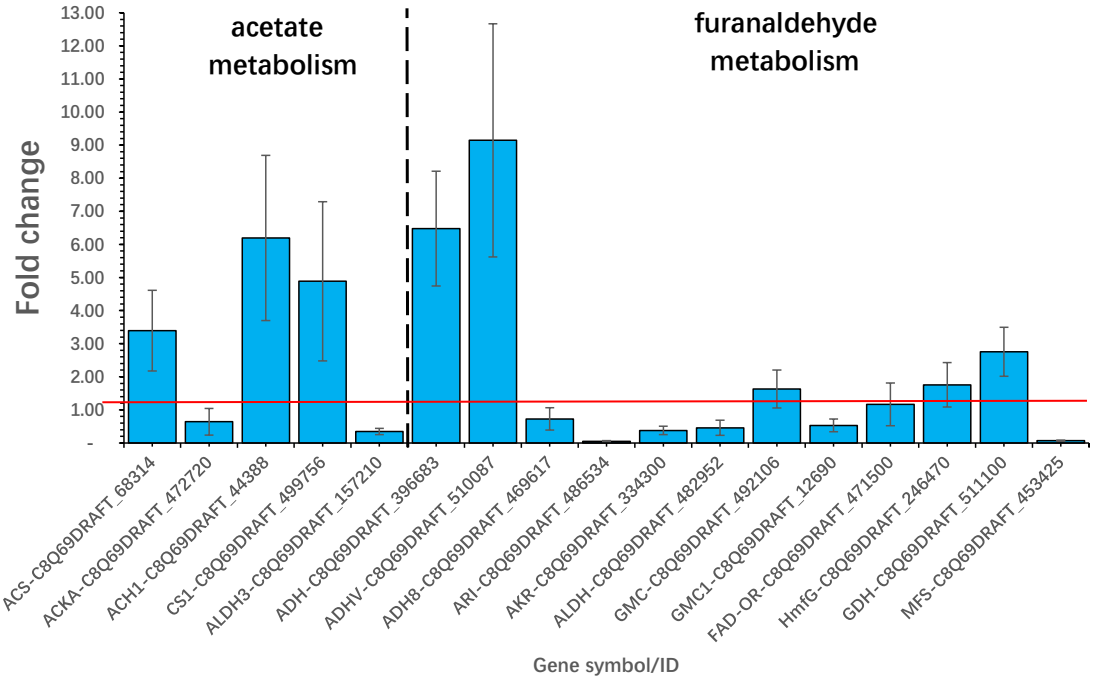


Figure 18 The expression level of the potential target genes by qRT-PCR for *P. variotii* CC004. Gene expression of *P. variotii* FN89 against all key target genes was assigned as biological control. Error bars represent the standard deviation of the average of three biological replicates.

In the furan aldehydes metabolism pathway, furan aldehydes undergo reduction to its alcohol form, HMF/furfuryl alcohol, facilitated by *ADH* (alcohol dehydrogenase), *AKR*/*ARI* (aldo-keto reductase/aldehyde reductase) and *GMC* (glucose–methanol–choline oxidoreductases). To elucidate the tolerance mechanism of furan aldehydes in isolate CC004, we conducted a transcriptional analysis of target genes involved in converting furan aldehydes into their alcohol form, including *AKR*, *ARI*, *ADH*, and *GMC*. So, we analyzed three *ADH* genes, including the *ADH* gene encoding alcohol dehydrogenase, the *ADHV* gene encoding alcohol dehydrogenase class V, and the *ADH8* gene encoding NADP-dependent alcohol dehydrogenase. As depicted in Figure 18, isolate CC004 exhibited a preferential to convert furan aldehydes into their alcohol form, as evidenced by the co-upregulation of *ADH* and *ADHV* genes by 6-fold greater. Conversely, while *AKR*/*ARI* pathways also facilitate aldehyde reduction to the alcohol form, they were deemed less preferred due to the downregulation of their expression levels. Additionally, both *GMC* and *GMC1* genes also displayed insignificant upregulation and downregulation expression levels, respectively, indicating a sluggish and slow reaction in oxidizing furan aldehydes into alcohol and/or acid forms in the presence of free oxygen. Transcriptional analysis of involved genes in furan aldehydes metabolism provided insights that isolate CC004 could metabolize furan aldehydes into furoic acid and then convert it to 2-oxoglutaric acid and then integrate into the TCA cycle.

Besides its role in the metabolic pathway, transporter genes, like *MFS* that encodes the major facilitator superfamily transporter, play a crucial role in the tolerance response to toxic compounds as an efflux pump to pump out toxic compounds from intracellular. However, this mechanism is aligned more with cell survival rather than detoxification. The Major Facilitator Superfamily (*MFS*) stands out as one of the most extensive assemblies of secondary active transporters, exhibiting remarkable selectivity in transporting a diverse range of substrates across membranes. As a result, it assumes a vital role in numerous stress responses under environmental stress. To delve into the functionality of the *MFS* gene of the tolerant phenotype, we conducted a transcriptional analysis, revealing a downregulation of the *MFS* gene. This outcome suggests that isolate CC004 employed a preference mechanism to detoxify inhibitors such as acetic acid, HMF, and formic acid. This detoxification occurs either by degrading these compounds into less toxic forms or utilizing them as a carbon source for further cellular metabolism during cultivation.

Glutamate-semialdehyde dehydrogenase (*GDH*) is the gene responsible for catalyzing the reduction of y-glutamyl phosphate to glutamate 5-semialdehyde in the proline biosynthesis pathway. We examined the *GDH* gene expression level to investigate its involvement in

tolerance phenotype. As depicted in Figure 18, we observed upregulation of the *GDH* gene by more than 2-*fold*, suggesting proline biosynthesis involved in tolerance phenotype to withdraw the negative impacts of inhibitors. In the synthesis of L-proline, two consecutive enzymatic steps are involved in the reduction reaction of L-glutamic acid to glutamate 5-semialdehyde. Initially, a y-glutamyl kinase activates the y-carboxyl group of glutamate. Subsequently, NADPH-dependent glutamate-semialdehyde dehydrogenase facilitates the reduction of glutamate to an aldehyde. Glutamate 5-semialdehyde spontaneously undergoes Schiff-base formation and cyclization, resulting in the formation of L-1-pyrroline-5-carboxylic acid. These intermediate compounds undergo reduction to proline through the action of an NADPH-dependent pyrroline-5-carboxylic acid reductase[234]. Previous studies in external enrichment of proline improved the tolerance of *C. acetobutylicum* ATCC 824 to lignocellulose-derived inhibitors. In addition, an engineered strain with enhanced proline biosynthesis capability exhibited improved cell growth and tolerance to furfural, acetic acid, and phenol[223, 235]. These results indicate the positive effects of proline biosynthesis in overcoming the drawback of lignocellulosic inhibitors. Proline functions are involved in stabilizing protein structures, maintaining cell membrane function, eliminating intracellular reactive oxygen species (ROS), and lowering the melting temperature of DNA. Proline can serve as an osmolyte, antioxidant, nutrient, or energy source, enhancing the response against osmotic and oxidative stress[224]. In summary, proline biosynthesis might have played a role in conferring tolerance of isolate CC004 to the high concentration of inhibitors cocktail in the cultivation environment.

In conclusion, isolate CC004 conferred its tolerance phenotype against major lignocellulose-derived inhibitors through preferentially converting furan aldehydes to its corresponding alcohols and acetic acid to acetyl CoA. These results indicated that the isolate CC004 could be utilized as a potential detoxification strain that could remove the potential inhibitors in biomass and enhance bioproduct productivity.

3.4 Conclusion

Our study demonstrated that the newly isolated strain *P. variotii* CC004 exhibited detoxification capability against main inhibitors in corncob residue without affecting the subsequent L-lactic acid fermentation process by *P. acidilactici* ZY271. New isolated *P. variotii* CC004 performed better acetic acid and HMF conversion than *P. variotii* FN89, indicating an efficient detoxification process. Furthermore, subsequent L-lactic acid fermentation of

detoxified hydrolysate by *P. variotii* CC004 resulted in 104.09 ± 0.31 g/L lactic acid accumulation and faster sugar utilization than that of *P. variotii* FN89. Transcriptional analysis revealed that the newly isolated strain *P. variotii* CC004 has a preferential to detoxify acetic acid through assimilation into acetyl-CoA and detoxify HMF through conversion into its less toxic alcohol form. These results exhibited the potential of *P. variotii* CC004 as a biodetoxification strain for corncob residue to optimize its utilization for L-lactic acid production.

Chapter 4 Conclusions and Perspectives

4.1 Conclusions

Lignocellulose biomass is a plentiful, sustainable and economical feedstock for biochemical production. The process involves pretreatment to release carbohydrates from the lignocellulosic matrix, followed by the hydrolysis step, and then fermentation using microorganisms. Various pretreatment methods, often employing mineral acids, facilitate hydrolysis. However, acid treatment yields inhibitory compounds alongside sugars, hampering desired biochemical production. These inhibitors include organic acids, furan derivatives and phenolic compounds originating directly from sugar degradation or the lignocellulosic polymer. They adversely affect cell physiology, diminishing viability, yield, and productivity. Pretreatment by dry dilute acid pretreatment could facilitate complete hydrolysis of the hemicellulose fraction, thereby enabling the subsequent enzymatic digestion of cellulose during hydrolysis and allowing high solid loading of feedstock without free water generation. The main drawback of dry dilute acid pretreatment is maintaining a high concentration of mixed inhibitors generated during the pretreatment process that are hindrances to fermentative strain. Therefore, removal efficiency, cost-effectiveness, and environmental impacts in removing high inhibitor concentrations in the lignocellulose hydrolysate play crucial roles in lignocellulose-based biorefinery. In addition, the current biodetoxification strain might indicate a drawback in lower cell viability and a slower removal rate during detoxification in high inhibitors containing lignocellulose hydrolysate. The research objective was to enhance the inhibitors removal capability of *P. variotii* FN89 in high inhibitors containing lignocellulose hydrolysate for sustainable, environmental-friendly, and high economic feasibility biorefineries of lignocellulosic biomass. The conclusions of this thesis are as follows:

1) Adaptive laboratory strategy in the solid substrate and submerged cultivation could enhance the detoxification performance of *P. variotii* FN89 in the high inhibitors containing lignocellulose hydrolysate. The resulting evolved strains, defined as strain AC70 and ZW70 were obtained from the evolutionary process using solid substrate and submerged cultivation, respectively. The evolved strains were proven more effective in removing high acetic acid and furan aldehydes containing wheat straw hydrolysate than the parental strain, indicating 50%, 55%, and 22% improvement of acetic acid, HMF and furfural degradation, respectively. In addition, the parental strain *P. variotii* FN89 exhibited severe growth inhibition during cultivation in the high inhibitors-containing medium.

2) The newly isolated strain from corncob residue, defined as *P. variotii* CC004, demonstrated its potential as a viable candidate for biodetoxification strain to degrade acetic acid, HMF, and formic acid in corncob residue hydrolysate. In addition, the newly isolated strain exhibited a higher degradation rate of acetic acid and HMF than the strain *P. variotii* FN89, indicating 40.39 and 25.64% enhancement, respectively. Utilizing bio-detoxified hydrolysate by the evolved strain AC70 and ZW70 and the newly isolated strain CC004 demonstrated a higher lactic acid accumulation by *P. acidilactici* ZY271.

3) Transcriptional analysis of evolved strains by qRT-PCR revealed that the evolved strain ZW70 exhibited a significant co-upregulation of target genes more than 3 folds, including *ACS*, *ACH1*, and *ACKA* in the acetic acid assimilation to acetyl-CoA and acetyl phosphate. In contrast, the newly isolated strain CC004 indicated a preferential to assimilate acetic acid into acetyl CoA by co-upregulation of *ACS* and *ACH1* genes more than 3 folds instead of acetyl phosphate. In addition, the evolved strains AC70 and ZW70 demonstrated a preferential to convert furan aldehydes into alcohol form by upregulation of *AKR* and *ADH* genes more than 3 folds. On the other hand, the newly isolated strain CC004 exhibited a preferential to convert furan aldehydes into alcohol form by upregulation of *ADH* and *ADHV* gene more than 5 folds. Our results indicated target genes involved in the acetic acid and furan aldehydes were significantly co-upregulated in the evolved strain obtained by ALE in submerged cultivation, indicating the submerged cultivation during evolutionary adaptation could better induce the tolerance of *P. variotii* FN89 toward high inhibitor concentrations. The biological detoxification of corncob residue hydrolysate could be performed by the newly isolated strain CC004 for subsequent cellulosic L-lactic acid fermentation. This research provides a potent biological detoxification method in corncob residue hydrolysate and its utilization for L-lactic acid biorefinery.

4) By enhancing the detoxification performance of the existing detoxifying fungus *P. variotii* FN89 through adaptive laboratory evolution and evaluating the detoxification ability of newly isolated strains CC004, this thesis offers microbial catalysts for the refinement of biofuels and biobased chemicals.

4.2 Prospects

Research works presented in this thesis provided a potential strategy to develop and isolate the robust detoxification strains to remove the main inhibitors in the lignocellulosic hydrolysates. The evolved strains *P. variotii* AC70 and ZW70 and newly isolated strain CC004 demonstrated their potential to detoxify the high inhibitor containing lignocellulosic hydrolysates, offering simple application, environmental-friendly, and cost-effectiveness for cellulosic L-lactic acid biorefinery. There are still many shortcomings in this study, and further research is needed:

1) Even if the concentration of phenolic compounds in lignocellulose hydrolysate is lower than the concentration of acetic acid and furan aldehydes, the compounds are known to be more toxic. Further investigation of the evolved strains *P. variotii* AC70 and ZW70 and newly isolated *P. variotii* CC004 should be studied in their phenolic compound degradation to optimize the productivity of fermentative strains.

2) The fermentation of the other bioproducts, such as bioethanol, lipid, and single-cell protein, should be performed after the biodetoxification by the evolved strains *P. variotii* AC70 and ZW70 and newly isolated *P. variotii* CC004 to ensure the detoxification strains could be utilized for the universal lignocellulose-based fermentation and don't cause negative impact for the subsequent fermentation.

3) Future characterization of *P. variotii* for a potential application in co-detoxification and fermentation of specific bioproduct conversion should be more effective, efficient, and beneficial for the economic feasibility of the biorefinery.

4) Indeed, a set of upregulated genes was identified, but the genotype-to-phenotype relationship remains to be determined. Some experimental verification of a complete transcriptional analysis and whole genome sequencing would be needed to provide in-depth knowledge of the molecular tolerance mechanism of *P. variotii*.

4.3 Innovations

The innovations of this thesis are as follows:

1) Common adaptive laboratory evolution is conducted only in one type of cultivation conditions. This thesis compares two different cultivation conditions during the evolutionary adaptation process to figure out the best cultivation method in ALE to enhance the detoxification performance of *P. variotii*.

2) The biological detoxification of corncob residue hydrolysate was less studied, which limits the utilization of corncob residue for the bioproduct refinery. This thesis provides an effective and efficient biological detoxification strategy to produce cellulosic L-lactic acid from the corncob residue hydrolysate.

This thesis provides a putative scheme of acetic acid and furan aldehydes metabolism pathway of *P. variotii* that hasn't been previously identified.

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List of abbreviations

Full name	Abbreviations
Ammonia Fiber Explosion	AFEX
Ammonia Recycle Percolation	ARP
Consolidated bioprocessing	CBP
Sulfuric acid	H_2SO_4
Sulphur dioxide	SO_2
5-hydroxymethylfurfural	HMF
Adenosine Triphosphate	ATP
Liquid hot water	LHW
Nicotinamide Adenine Dinucleotide	NADH
Nicotinamide Adenine Dinucleotide Phosphate	NADPH
Reactive oxygen species	ROS
Deoxyribonucleic Acid	DNA
Ribonucleic Acid	RNA
Polyethyleneimine	PEI
Waste house wood	WHW
High-pressure liquid chromatography	HPLC
High-pressure liquid chromatography-ultraviolet-tandem mass spectrometry	HPLC-UV-ms/MS
Lactic acid	LA
Pediococcus acidilactici ZY271	*P. acidilactici* ZY271
Paecilomyces variotii	*P. variotii*
Paecilomyces variotii FN89	*P. variotii* FN89
Adaptive laboratory evolution	ALE
Saccharomyces cerevisiae	*S. cerevisiae*
Visualizing evolution in real-time	VERT
Dry dilute acid pretreatment	DDAP
Quantitative real-time polymerase chain reaction	qRT-PCR
China General Microbiological Culture Collection	CGMCC

Potato dextrose agar	PDA
Synthetic medium	SM
Yeast extract	YE
Potassium dihydrogen phosphate	KH_2PO_4
Magnesium sulfate heptahydrate	$MgSO_4.7H_2O$
Ammonium sulphate	$(NH_4)_2SO_4$
Calcium chloride	$CaCl_2$
Hydrochloric acid	HCl
Man-Rogosa-Sharp	MRS
Dipotassium hydrogen phosphate trihydrate	$K_2HPO_4.3H_2O$
Manganese sulfate monohydrate	$MnSO_4.H_2O$
Revolutions per minute	rpm
Air volume/culture volume/min	vvm
National Renewable Energy Laboratory	NREL
Potential hydrogen/power of hydrogen ion	pH
Internal transcribe spacer	ITS
Volume to volume	v/v
Weight to weight	w/w
Fold change	FC
genomic Deoxyribonucleic Acid	gDNA
Dry cell weight	DCW
Standard deviation	SD
Tricarboxylic acid cycle	TCA cycle
Adenosine monophosphate	AMP
Adenosine diphosphate	ADP
acetyl-CoA synthetase	*acs*
acetate kinase	*ack*
acetate hydrolase	ach
2,5-furan-dicarboxylic acid	FDCA
Sodium chloride	NaCl
Ribosomal Deoxyribonucleic Acid	rDNA
Corn saccharification liquid	CR
Yeast hydrolysate	YH

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