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Tolerance and transcriptional analysis of *Corynebacterium glutamicum* on biotransformation of toxic furaldehyde and benzaldehyde inhibitory compounds

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Abstract

Furaldehydes and benzaldehydes are among the most toxic inhibitors from lignocellulose pretreatment on microbial growth and metabolism. The bioconversion of aldehyde inhibitors into less toxic alcohols or acids (biotransformation) is the prerequisite condition for efficient biorefinery fermentations. This study found that *Corynebacterium glutamicum* S9114 demonstrated excellent tolerance and biotransformation capacity to five typical aldehyde inhibitors including two furaldehydes: 2-furaldehyde (furfural), 5-(hydroxymethyl)-2-furaldehyde, and three benzaldehydes: 4-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde (vanillin), and 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde). Transcription levels of 93 genes hypothesized to be responsible for five aldehydes biotransformation were examined by qRT-PCR. Multiple genes showed significantly up-regulated expression against furaldehydes or benzaldehydes. Overexpression of *CGS9114_RS01115* in *C. glutamicum* resulted in the increased conversion of all five aldehyde inhibitors. The significant oxidoreductase genes responsible for each or multiple inhibitors biotransformation identified in this study will serve as a component of key gene device library for robust biorefinery fermentation strains development in the future biorefinery applications.

Keywords *Corynebacterium glutamicum* S9114 · Furaldehydes · Benzaldehydes · Biotransformation · Transcriptional response

Introduction

Pretreatment is the crucial step for overcoming biorecalcitrance of lignocellulose to release fermentable sugars by enzymatic hydrolysis. In the harsh pretreatment process, various inhibitors are generated and severely inhibit the cell growth and metabolism of biorefinery fermentation strains in consequent fermentation step [5]. The inhibitors include furaldehydes: 2-furylaldehyde (furfural) and 5-(hydroxymethyl)-2-furaldehyde (HMF) from dehydration of pentose and hexose; weak organic acids: formic acid, acetic acid, and levulinic acid from carboxylate group hydrolysis or furans oxidation; benzaldehydes: 4-hydroxybenzaldehyde (HBA), 4-hydroxy-3-methoxybenzaldehyde (vanillin), and 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde) from lignin degradation [10, 22]. Furaldehydes and benzaldehydes are the most toxic inhibitors to most fermentation microorganisms [8, 20, 40, 41, 44] and their effective removal is the prerequisite condition for efficient biorefinery fermentations. Among the various detoxification methods, biological transformation of furaldehydes into less toxic alcohols and acids (biodetoxification) by the special biodetoxification microorganisms provides the most efficient way with significant advantages of fast, complete, and less waste water generation [12, 16, 21, 37, 43]. However, biotransformation of benzaldehydes from pretreated lignocellulose biomass is not as efficient as furaldehydes because of their low water solubility and hydrophobicity [7, 31, 43]. Therefore, screening of robust fermentation strains to various aldehyde inhibitors is an important option in fermentation step for

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converting the furaldehydes and benzaldehydes-containing lignocellulose feedstock into target products.

Corynebacterium glutamicum is a biotin auxotrophic Gram-positive bacterium for production of amino acids and biochemicals [27, 32, 39]. *C. glutamicum* had been found to be highly tolerant to the aldehydes-containing lignocellulose system [38] through converting furfural into the furfuryl alcohol and furoic acid by alcohol and aldehyde dehydrogenases [23, 32, 33], and vanillin into vanillic acid by vanillin dehydrogenase [4, 6]. *C. glutamicum* is also capable of assimilating vanillin, vanillic acid, ferulic acid, cumaric acid, and 4-hydroxybenzoic acid into its central metabolism [13, 28–30]. However, the molecular biotransformation metabolism of converting the main furaldehydes and benzaldehydes inhibitors into less toxic alcohols or acids by *C. glutamicum* is still not clear.

In this study, the tolerance and biotransformation of *C. glutamicum* S9114, a widely applied industrial strain, to the five aldehyde inhibitors including furfural, HMF, HBA, vanillin, and syringaldehyde were examined and 93 genes hypothesized to be responsible for aldehyde conversion were identified by transcriptional analysis. Genes involved biotransformation pathways of two furaldehydes and three benzaldehydes inhibitors were suggested based on the inhibitor conversion results, transcriptional analysis, and relevant studies. Overexpression of several important genes in *C. glutamicum* resulted in the increased conversion of furaldehydes or benzaldehydes. The results provide the first comprehensive insight into the biotransformation mechanism of aldehyde inhibitors in *C. glutamicum* and the valuable genetic resources for metabolic engineering of robust fermenting strain development in future biorefinery applications.

Materials and methods

Strains, plasmids, media, and culture conditions

Microbial strains and plasmids used in this study were shown in Table S2. *C. glutamicum* S9114 was obtained from Shanghai Industrial Institute of Microbiology (SIIM), Shanghai, China with the storage number of SIIM B460. *C. glutamicum* S9114 has the same origin to *C. glutamicum* CICC 20935 in the deposit of China Center of Industrial Culture Collection (CICC) with the registration number of CICC 20935. The whole genome sequence of *C. glutamicum* S9114 is obtained at GenBank under the accession number of AFYA00000000 [19].

The culture media used include: (1) LB agar medium (g/L), yeast extract 5, tryptone 10, NaCl 5, and agar 15; (2) pre-culture medium (g/L), glucose 25, corn steep liquor 25, KH_2PO_4 1.5, $MgSO_4{\cdot}7H_2O$ 1.2, urea 2.5, $FeSO_4{\cdot}7H_2O$ 0.0037, and $MnSO_4{\cdot}H_2O$ 0.0022; (3) seed culture medium (g/L), glucose 25, corn steep liquor 5, KH_2PO_4 1.5, $MgSO_4{\cdot}7H_2O$ 1.2, urea 2.5, $FeSO_4{\cdot}7H_2O$ 0.0037, and $MnSO_4{\cdot}H_2O$ 0.0022; and (4) fermentation medium (g/L), glucose 60, corn steep liquor 0.5, KH_2PO_4 1, $MgSO_4{\cdot}7H_2O$ 1.2, urea 3, $FeSO_4{\cdot}7H_2O$ 0.0037, and $MnSO_4{\cdot}H_2O$ 0.0022. All media sterilized at 115 °C for 20 min. $FeSO_4$ and $MnSO_4$ solutions prepared freshly to avoid the oxidation and sterilized by 0.22-μm bacterial filter.

One vial of *C. glutamicum* S9114 was used to streak onto LB agar plate and cultured for 36 h at 30 °C. A single colony was transferred into 30 mL of pre-culture medium in 250 mL Erlenmeyer flask and incubated for 10 h at 30 °C, 200 rpm and pH 7.0. 5% (v/v) of the culture was transferred into seed culture medium and incubated for 8 h at 30 °C, 200 rpm, and pH 7.0. The seed culture was used as the inoculum in all fermentation experiments. *Escherichia coli* BL21 was cultured on LB medium with 50 μg/mL of kanamycin addition if needed.

Inhibitor tolerance and biotransformation evaluation

Inhibitor tolerance experiment was carried out in the fermentation medium with gradient concentration of aldehydes addition. 5% (v/v) of the seed culture was inoculated into 30 mL of fermentation medium at 250 mL flask and cultured at 30 °C, 200 rpm. pH was adjusted to 7.0 with the addition of 20% urea at regular intervals. After 36 h, samples were collected for cell growth and glutamic acid concentration measurement. All experiments were done in duplicates.

Inhibitor biotransformation experiment was conducted in the fermentation medium amended by separate addition of 10.3 mM of furfural, 5.6 mM of HMF, 2.4 mM of HBA, 1.3 mM of vanillin, or 1.1 mM of syringaldehyde. 10% (v/v) of the seed culture was inoculated into 30 mL of fermentation medium at 250 mL flask, 30 °C and 200 rpm. pH was adjusted to 7.0 with the addition of 20% urea at regular intervals. Culture samples were periodically collected for aldehydes and its corresponding metabolites concentration detection. All experiments were done in duplicates.

Analysis

Cell growth was determined by measuring the optical density at 600 nm (OD_{600}) using DU800 spectrophotometer (Beckman Coulter Inc., USA). Samples collected during fermentation were centrifuged at 12,000 rpm for 5 min, and then, the supernatants were filtered through a 0.22-μm filter before analysis.

Glucose and glutamic acid were monitored with SBA Biosensor 40D (Shandong Academy of Sciences, Jinan, China). Furfural, furfuryl alcohol, HMF, 5-hydroxymethylfurfuryl

alcohol (HMF alcohol), 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, 4-hydroxybenzoic acid, vanillin, vanillyl alcohol, vanillic acid, syringaldehyde, syringic alcohol, and syringic acid were analyzed on reverse-phase HPLC (LC-20AT, Japan), equipped with a YMC-Pack ODS-A column (YMC, Tokyo, Japan) and an SPD-20A UV detector (Shimadzu, Kyoto, Japan) at the column temperature of 35 °C. Furfural and furfuryl alcohol were analyzed using 50% acetonitrile solution as the mobile phase at 1.0 mL/min and the detector wavelength of 220 nm. HMF and HMF alcohol were analyzed at the mobile phase rate of 0.6 mL/min and the detector wavelength of 230 nm. The gradient procedure applied as following: the initial flow phase was composed by pure water (pump A) and acetonitrile (pump B) at a ratio of 95–5%; first, acetonitrile was increased from 5 to 100% over 0–15 min then decreased from 100 to 5% over 15–20 min, and finally used at 5% over 20–30 min. 4-Hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, 4-hydroxybenzoic acid, vanillin, vanillyl alcohol, vanillic acid, syringaldehyde, syringic alcohol, and syringic acid were analyzed using 100% acetonitrile solution (pump A) and 0.1% formic acid (pump B) at a ratio of 90–10% as the mobile phase at 1.0 mL/min and the detector wavelength of 270 nm. 2-Furoic acid (furoic acid) and 5-hydroxymethylfuroic acid (HMF acid) were determined on HPLC (LC-20D, Japan) equipped with a refractive index detector RID-10A (Shimadzu, Kyoto, Japan) and an Aminex HPX-87H column (BioRad, Hercules, CA, USA) operated at 65 °C with 0.6 mL/min of 5 mM H_2SO_4 as the mobile phase.

qRT-PCR analysis

Quantitative real-time PCR (qRT-PCR) was conducted in the pre-culture medium amended by separate addition of 15.6 mM of furfural, 11.9 mM of HMF, 8.2 mM of HBA, 6.6 mM of vanillin, or 0.5 mM of syringaldehyde. The pre-culture medium without inhibitor addition was used as control. Cells were separately harvested at 4 h after furaldehydes treatment and 8 h after benzaldehydes treatment, centrifuged at 8000 rpm for 5 min at 4 °C, then stored at − 80 °C for subsequent analysis.

The total RNA was extracted using Trizol reagent (RNAiso Plus, Takara, Otsu, Japan) after the cell mass was grinded under liquid nitrogen. The RNA integrity was assessed by gel electrophoresis and the RNA quantity was determined by BioMate 3S UV–visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription reactions were carried out using ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. For each qRT-PCR reaction, an SYBR Green Real-Time PCR Master Mix kit (Toyobo, Osaka, Japan) was used and the PCR reaction was run on a BioRad CFX 96 system (BioRad, Hercules, CA, USA).

The primers for qRT-PCR analysis were listed in Table S1. The amplification length of all test genes ranged from 80 to 180 bp. The 16S rDNA gene *CGS9114_RS11955* of *C. glutamicum* S9114 was used as an internal control to normalize for difference in total RNA quantity for its stable expression under all the experimental and control groups. qRT-PCR reactions were prepared in a total volume of 20 µL containing 4 µM each gene-specific primer, 10 µL 2 × SYBR Green Mix (Toyobo, Osaka, Japan), and 2 µL cDNA (diluted 1:9). The cycle conditions were set as following: 95 °C for 1 min, followed 40 cycles at 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s; a final melting curve step by heating from 65 °C to 95 °C with a speed of 0.5 °C per 5 s. Transcription levels of interesting genes were quantified using the formula $2^{-\Delta\Delta Ct}$. The differentially expressed genes were determined with a selection threshold of fold change ≥ 2.0 (up-regulation) or ≤ 0.5 (down-regulation). Most significantly, up-regulated genes were determined with a selection threshold of fold change ≥ 5.0 (for furaldehydes) or ≥ 3.0 (for benzaldehydes).

Gene expression in *C. glutamicum* and inhibitor conversion evaluation

The genomic DNA of *C. glutamicum* S9114 was extracted using TIANamp Bacterial DNA Kit (Tiangen Biotech Co., Beijing, China). The primers used for each target gene amplification and the constructed recombinant plasmids in this study were listed in Table S2. The overexpression plasmid pEFTUmob used in this study was constructed by replacing the Trc promoter of pTRCmob [15] with a strong promoter of the gene-encoding elongation factor TU (EFTU) [2]. Each target oxidoreductase gene was amplified and inserted into *Xba*I/*Pst*I-treated pEFTUmob by T4 ligase (Takara, Otsu, Japan) or seamless cloning kit (Hanheng Biotech Co., Nanjing, China). The recombinant plasmid was transformed into *C. glutamicum* S9114 following the method of van der Rest [34].

The cell growth and inhibitor conversion evaluation of recombinant *C. glutamicum* were conducted in the fermentation medium with the addition of 17 mM furfural, 17 mM HMF, 6.6 mM HBA, 6.6 mM vanillin, or 0.8 mM syringaldehyde, respectively. 5% (v/v) of the seed culture was inoculated into 10 mL of fermentation medium at 100 mL flask, 30 °C, and 200 rpm. Culture samples were collected for cell growth and inhibitor concentration detection at 8 h, 8 h, 16 h, 16 h, and 12 h by furfural, HMF, HBA, vanillin, and syringaldehyde, respectively. All experiments were done in duplicates.

Results and discussion

Inhibitor tolerance and biotransformation performance of *C. glutamicum* S9114

The tolerance of *C. glutamicum* S9114 to the five aldehyde inhibitors (furfural, HMF, HBA, vanillin, and syringaldehyde) was examined under the practical inhibitors concentration range of lignocellulose hydrolysate as determined in our previous study [35]. For the two furaldehydes, the cell growth of *C. glutamicum* S9114 showed negligible effect until to a very high inhibitor concentration (26.0 mM of furfural, almost fourfold greater than that of the corn stover hydrolysate (CSH); 23.8 mM of HMF, more than fivefold greater than that of the CSH), but the glutamic acid productivity decreased steadily with increasing aldehyde concentrations (Fig. 1a, b). For the three benzaldehydes, HBA showed a limited inhibition on cell growth and glutamic acid productivity even at high concentration (16.4 mM of HBA, more than two orders of magnitude greater than that of the CSH) (Fig. 1c). Vanillin

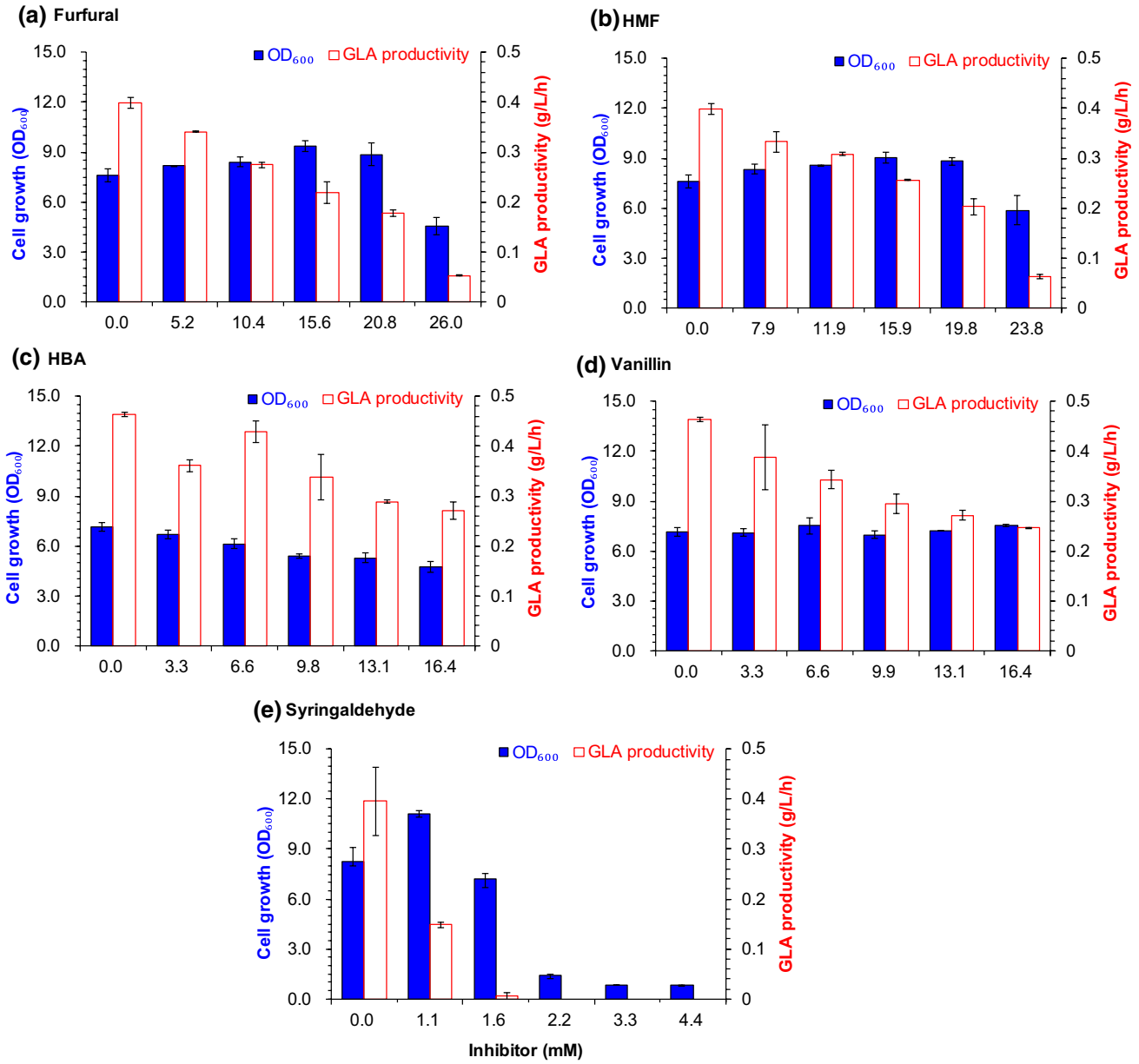


Fig. 1 Tolerance of *C. glutamicum* S9114 to lignocellulose-derived aldehyde inhibitors. **a** Furfural; **b** HMF; **c** HBA; **d** vanillin; **e** syringaldehyde. *GLA* glutamic acid. Conditions: pH 7.0, 30 °C and 200 rpm for 36 h in synthetic medium. Mean values were presented with error bars representing at least two standard deviations

also showed negligible inhibition on the cell growth at high concentration (16.4 mM, more than 13-folds greater than that of the CSH), but glutamic acid productivity was reduced by 47.0% comparing to that of the control (Fig. 1d). Different from HBA and vanillin, syringaldehyde showed strong inhibition on *C. glutamicum* S9114, resulting in the complete cease of cell growth and glutamic acid productivity by only 1.7–2.2 mM of syringaldehyde (threefold greater than that of the CSH) (Fig. 1e). Though the glutamic acid productivity was inhibited by 87.0%, 84.3%, 41.6%, 47.0%, or 96.3% under the maximum concentrations of individual inhibitors (26.0 mM of furfural, 23.8 mM of HMF, 16.4 mM of HBA, 16.4 mM of vanillin, or 1.7 mM of syringaldehyde), the cell growth inhibited by 40.1%, 22.7%, 34.0%, 0%, or 35.3%, respectively. In general, these typical aldehyde inhibitors showed more inhibition on the glutamic acid productivity than the cell growth of *C. glutamicum* S9114.

The inhibitor biotransformation performance of *C. glutamicum* S9114 was investigated under the inhibitor concentration higher than the practical concentration of the corn stover hydrolysate [35]. Furfural and HMF were quickly reduced to furfuryl alcohol and HMF alcohol, and then oxidized to furoic acid and HMF acid, respectively (Fig. 2a,

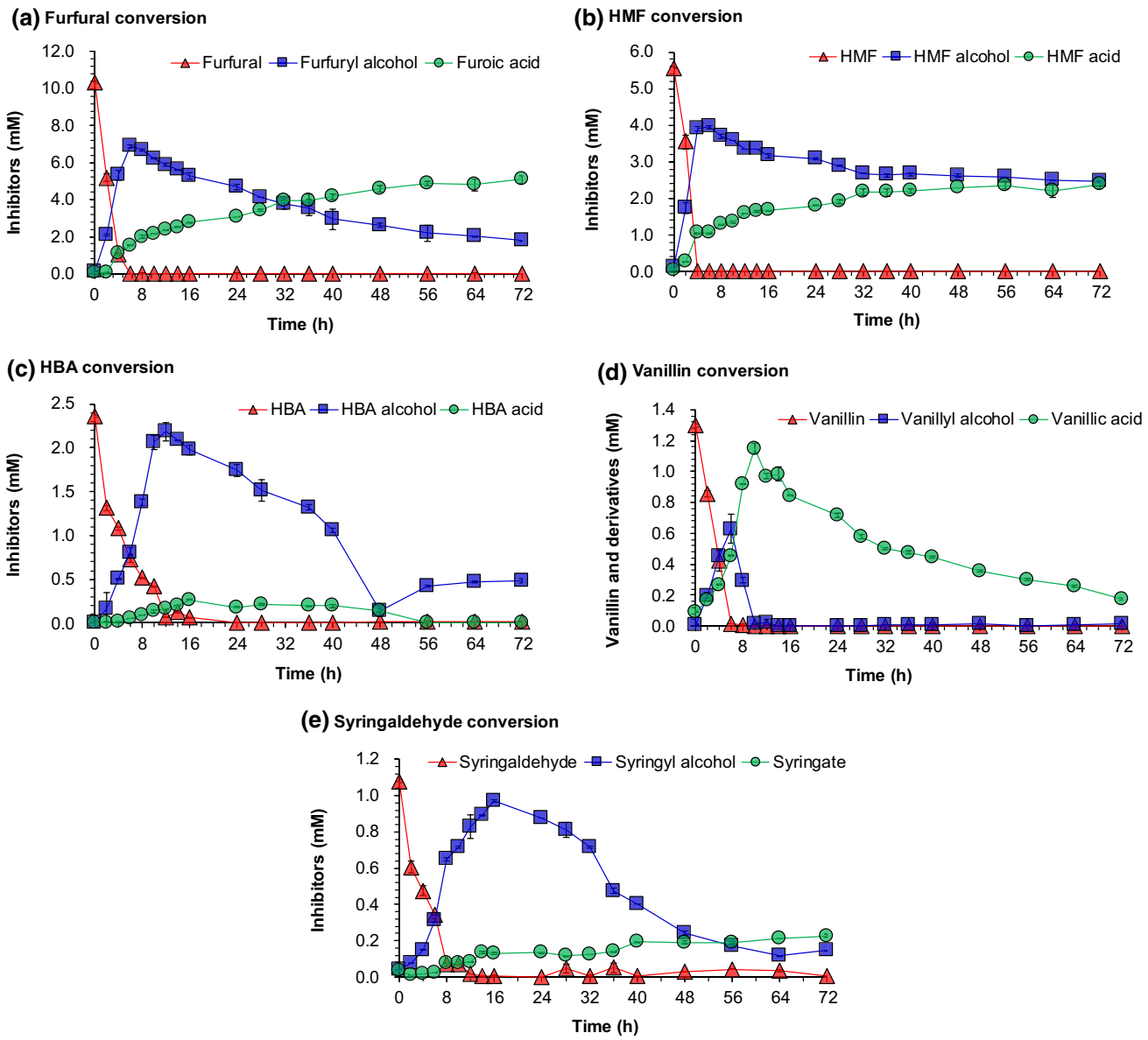
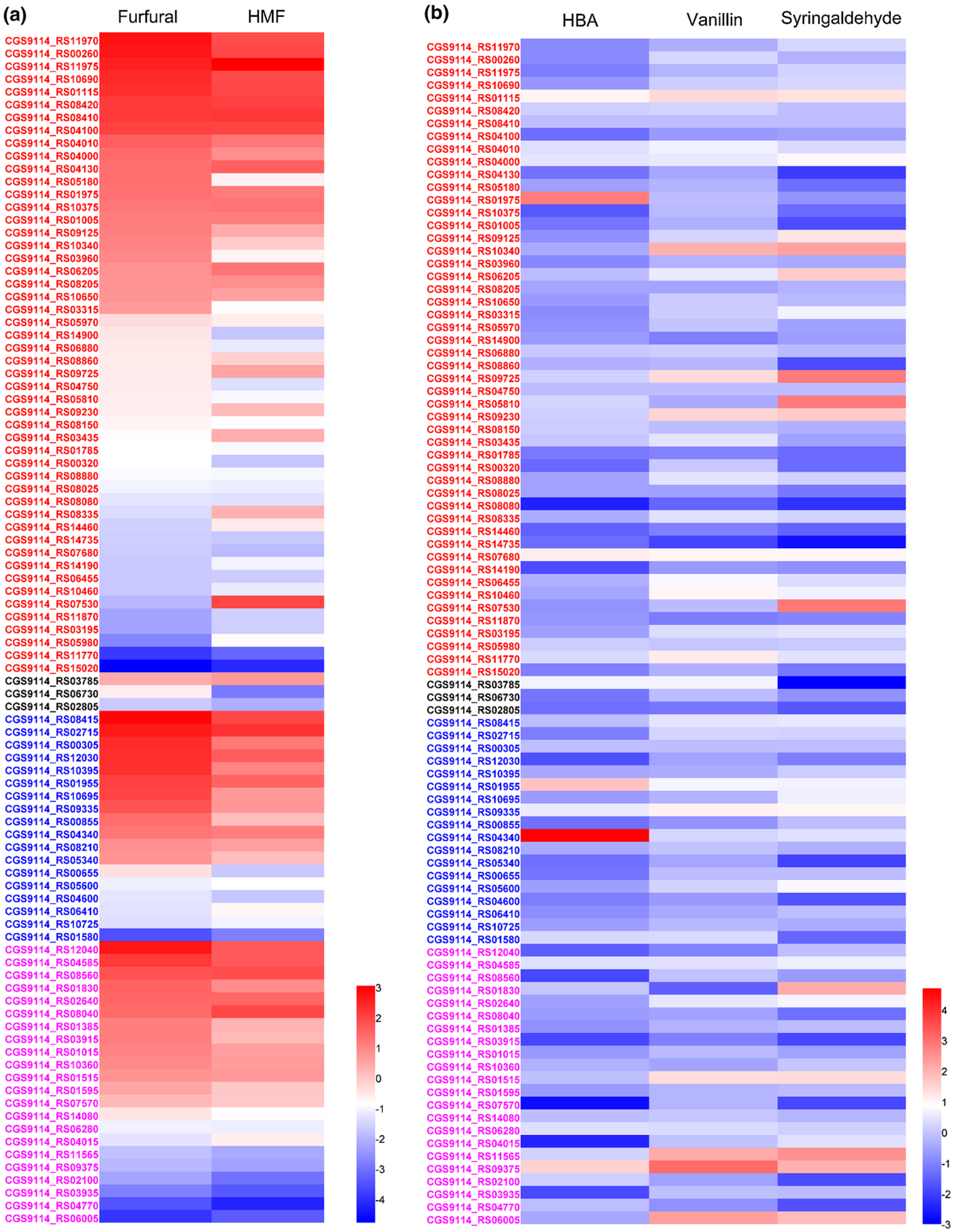


Fig. 2 Inhibitor biotransformation by *C. glutamicum* S9114. **a** Furfural 10.3 mM, **b** HMF 5.6 mM, **c** HBA 2.4 mM, **d** vanillin 1.3 mM, **e** syringaldehyde 1.1 mM. Conditions: pH 7.0, 30 °C, and 200 rpm for 72 h in synthetic medium. Mean values were presented with error bars representing at least two standard deviations


(a)
Furfural
HMF
CGS9114_RS11970
CGS9114_RS00260
CGS9114_RS11975
CGS9114_RS10690
CGS9114_RS01115
CGS9114_RS08420
CGS9114_RS08410
CGS9114_RS04100
CGS9114_RS04010
CGS9114_RS04000
CGS9114_RS04130
CGS9114_RS05180
CGS9114_RS01975
CGS9114_RS10375
CGS9114_RS01005
CGS9114_RS09125
CGS9114_RS10340
CGS9114_RS03960
CGS9114_RS06205
CGS9114_RS08205
CGS9114_RS10650
CGS9114_RS03315
CGS9114_RS05970
CGS9114_RS14900
CGS9114_RS06880
CGS9114_RS08860
CGS9114_RS09725
CGS9114_RS04750
CGS9114_RS05810
CGS9114_RS09230
CGS9114_RS08150
CGS9114_RS03435
CGS9114_RS01785
CGS9114_RS00320
CGS9114_RS08880
CGS9114_RS08025
CGS9114_RS08080
CGS9114_RS08335
CGS9114_RS14460
CGS9114_RS14735
CGS9114_RS07680
CGS9114_RS14190
CGS9114_RS06455
CGS9114_RS10460
CGS9114_RS07530
CGS9114_RS11870
CGS9114_RS03195
CGS9114_RS05980
CGS9114_RS11770
CGS9114_RS15020
CGS9114_RS03785
CGS9114_RS06730
CGS9114_RS02805
CGS9114_RS08415
CGS9114_RS02715
CGS9114_RS00305
CGS9114_RS12030
CGS9114_RS10395
CGS9114_RS01955
CGS9114_RS10695
CGS9114_RS09335
CGS9114_RS00855
CGS9114_RS04340
CGS9114_RS08210
CGS9114_RS05340
CGS9114_RS00655
CGS9114_RS05600
CGS9114_RS04600
CGS9114_RS06410
CGS9114_RS10725
CGS9114_RS01580
CGS9114_RS12040
CGS9114_RS04585
CGS9114_RS08560
CGS9114_RS01830
CGS9114_RS02640
CGS9114_RS08040
CGS9114_RS01385
CGS9114_RS03915
CGS9114_RS01015
CGS9114_RS10360
CGS9114_RS01515
CGS9114_RS01595
CGS9114_RS07570
CGS9114_RS14080
CGS9114_RS06280
CGS9114_RS04015
CGS9114_RS11565
CGS9114_RS09375
CGS9114_RS02100
CGS9114_RS03935
CGS9114_RS04770
CGS9114_RS06005
3
2
1
0
-1
-2
-3
-4
(b)
HBA
Vanillin
Syringaldehyde
CGS9114_RS11970
CGS9114_RS00260
CGS9114_RS11975
CGS9114_RS10690
CGS9114_RS01115
CGS9114_RS08420
CGS9114_RS08410
CGS9114_RS04100
CGS9114_RS04010
CGS9114_RS04000
CGS9114_RS04130
CGS9114_RS05180
CGS9114_RS01975
CGS9114_RS10375
CGS9114_RS01005
CGS9114_RS09125
CGS9114_RS10340
CGS9114_RS03960
CGS9114_RS06205
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CGS9114_RS03315
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CGS9114_RS14460
CGS9114_RS14735
CGS9114_RS07680
CGS9114_RS14190
CGS9114_RS06455
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CGS9114_RS03785
CGS9114_RS06730
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CGS9114_RS08415
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CGS9114_RS00305
CGS9114_RS12030
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CGS9114_RS00855
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CGS9114_RS06410
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CGS9114_RS12040
CGS9114_RS04585
CGS9114_RS08560
CGS9114_RS01830
CGS9114_RS02640
CGS9114_RS08040
CGS9114_RS01385
CGS9114_RS03915
CGS9114_RS01015
CGS9114_RS10360
CGS9114_RS01515
CGS9114_RS01595
CGS9114_RS07570
CGS9114_RS14080
CGS9114_RS06280
CGS9114_RS04015
CGS9114_RS11565
CGS9114_RS09375
CGS9114_RS02100
CGS9114_RS03935
CGS9114_RS04770
CGS9114_RS06005
4
3
2
1
0
-1
-2
-3

◂**Fig. 3** Transcription levels for selected relevant genes in *C. glutamicum* S9114 in response to **a** two furaldehyde inhibitory compounds and **b** three benzaldehyde inhibitory compounds. Quantitative expression level is $\log_2$ transformed from raw fold changes against that without aldehyde addition sample. Red indicates up-regulated expression and blue for down-regulated expression as indicated by a color bar at the figure right. The candidate genes are listed on the figure left and the color from top to bottom indicates different categories of genes: red, ADH genes; black, AKR/ARI genes; blue, ALDH genes; and purple, other oxidoreductases without detailed functional annotation genes (color figure online)

b). No further decrease of the acids was observed, indicating that furoic acid and HMF acid were the relatively stable metabolites of *C. glutamicum* S9114 in the experimental range. Similar to the two furaldehydes conversion, the three benzaldehydes were also quickly converted into the corresponding alcohols, and then oxidized into corresponding acids (Fig. 2c,e). Contrary to furaldehydes conversion, 4-hydroxybenzoic acid and vanillic acid continued to decrease, indicating that the two acids maybe further assimilated via a β-ketoadipate route into TCA cycle in *C. glutamicum* S9114 [9, 28, 30]. Syringic alcohol declined quickly and syringic acid formation only increased slightly, indicating the considerable syringic acid formed might also be assimilated in the similar way to HBA acid and vanillic acid (Fig. 2e). The self-decomposition of each aldehyde was negligible within the time of completely biotransformation by *C. glutamicum* S9114 (data not shown).

These results suggested that *C. glutamicum* S9114 behaved a strong tolerance to furaldehyde and benzaldehyde inhibitors by converting aldehydes to corresponding alcohols and acids with lessened toxicity. The benzaldehydes could be further assimilated into an ultimate degradation in *C. glutamicum*. The biotransformation performance of aldehyde inhibitors in *C. glutamicum* S9114 is not only similar to some biodetoxification microorganisms which are specially used for lignocellulose inhibitors biodetoxification such as *Cupriavidus basilensis* HMF14 and *Amorphotheca resinae* ZN1, but also similar to some fermentation strains which are able to transform the inhibitors in the fermentation biorefinery step such as *Saccharomyces cerevisiae* and *Trichosporon cutaneum* [11, 16, 35, 36].

Transcriptional response of *C. glutamicum* to furaldehydes and benzaldehydes

Oxidoreductase is responsible for reducing aldehydes into less toxic alcohols or oxidizing into acids in many biodetoxification microbes [18, 24, 36, 40]. This study screened 80 oxidoreductase genes based on the whole genome annotation of *C. glutamicum* S9114 (GenBank: AFYA00000000) [19] (Table S1). Another 13 hypothetical oxidoreductase genes were also selected based on the Clusters of Orthologous Groups of proteins (COG) analysis (http://www.ncbi.nlm.nih.gov/COG/) (Table S1). Enzymes encoded by these selected total 93 genes included 50 NAD(P)(H)-alcohol dehydrogenases (ADH), 3 aldo–keto reductase/aldehyde reductases (AKR/ARI), 18 aldehyde dehydrogenases (ALDH), and 22 other oxidoreductases without detailed functional annotation. ADH and AKR/ARI were hypothesized to be responsible for reversible conversion of furaldehydes and benzaldehydes to the corresponding alcohols and vise versa. ALDH was hypothesized to be responsible for the oxidation of aldehydes to the corresponding acids. The transcriptional response of the 93 genes to furaldehydes and benzaldehydes inhibitors was measured by qRT-PCR, as shown in Fig. 3.

Under the stress of two furaldehydes (Fig. 3a), 18 ADH genes, 10 ALDH genes, and 10 other oxidoreductase genes were up-regulated and 26 genes were down-regulated in response to furfural stress. For HMF, 17 ADH genes, 7 ALDH genes, and 6 other oxidoreductase genes were up-regulated and 25 genes were down-regulated. Among these up-regulated genes, 14 ADH genes, 7 ALDH genes, and 6 other oxidoreductase genes were shared by furfural and HMF (Table 1), suggesting that furfural and HMF may induce the similar oxidoreductase genes to convert the furaldehydes into corresponding alcohols or acids in *C. glutamicum* S9114.

Under the stress of three benzaldehydes (Fig. 3b), 3 ADH genes, 2 ALDH genes, and 1 other oxidoreductase genes were up-regulated and 22 genes were down-regulated in response to HBA stress. For vanillin, 7 ADH genes, 1 ALDH genes, and 4 other oxidoreductase genes were up-regulated and 4 genes were down-regulated. For syringaldehyde, 10 ADH genes, 1 ALDH genes, and 5 other oxidoreductase genes were up-regulated and 22 genes were down-regulated. Among these up-regulated genes, 5 ADH genes, 1 ALDH genes and 4 other oxidoreductase genes were shared by at least two benzaldehydes (Table 1).

Overall, the transcriptional response of *C. glutamicum* S9114 to furaldehydes and benzaldehydes shows that the biotransformation of each aldehyde is likely to be the synergistic result of multiple genes' interactions. The gene expression response and biotransformation mechanism were apparently different between the two furaldehydes and three benzaldehydes. The number of the significantly up-regulated expression genes responding to benzaldehydes was less than that responding to furan aldehydes, which is consistent with the performance that the actual biotransformation rate of benzaldehydes was lower than that of furan aldehydes. In addition, the activity of the enzymes responsible for benzaldehydes might be not as high as the enzymes for furan aldehydes in the same fermentation condition; thus, higher expression of the enzymes is required for improving the benzaldehydes biotransformation rate. Two ADH

Table 1 Up-regulated genes involved in furaldehydes' and benzaldehydes' conversion in *C. glutamicum* S9114

Genes	Genes ID	Functional annotation	Fold change				
			Furfural	HMF	HBA	Vanillin	Syringaldehyde
ADH	CGS9114_RS00260	Alcohol dehydrogenase	7.12 ± 1.05	4.31 ± 0.64	–	–	–
	CGS9114_RS10690	Alcohol dehydrogenase	5.92 ± 0.63	4.20 ± 1.43	–	–	–
	CGS9114_RS04130	Alcohol dehydrogenase	2.81 ± 0.52	3.32 ± 1.92	–	–	–
	CGS9114_RS08205	Alcohol dehydrogenase	–	2.02 ± 0.66	–	–	–
	CGS9114_RS01115	Alcohol dehydrogenase	5.54 ± 2.16	4.84 ± 0.76	2.25 ± 1.14	2.25 ± 1.14	2.69 ± 1.11
	CGS9114_RS08420	Benzyl alcohol dehydrogenase	4.84 ± 0.97	4.56 ± 2.10	–	–	–
	CGS9114_RS04100	Short-chain dehydrogenase	4.55 ± 0.77	4.43 ± 1.80	–	–	–
	CGS9114_RS03960	Short-chain dehydrogenase	2.16 ± 0.49	–	–	–	–
	CGS9114_RS01975	Short-chain dehydrogenase	2.77 ± 1.18	2.49 ± 0.58	7.82 ± 0.27	–	–
	CGS9114_RS10375	Inositol 2-dehydrogenase	2.60 ± 0.91	2.69 ± 1.37	–	–	–
	CGS9114_RS04010	Inositol 2-dehydrogenase	2.57 ± 1.62	2.58 ± 0.78	–	–	–
	CGS9114_RS11970	Inositol 2-dehydrogenase	7.42 ± 0.61	4.04 ± 1.04	–	–	–
	CGS9114_RS11975	Inositol 2-dehydrogenase	6.37 ± 2.26	9.07 ± 3.74	–	–	–
	CGS9114_RS10340	Inositol 2-dehydrogenase	2.25 ± 0.69	–	–	3.27 ± 2.42	5.37 ± 4.70
	CGS9114_RS04000	Inositol 2-dehydrogenase	2.92 ± 0.12	2.01 ± 0.73	–	–	2.17 ± 0.73
	CGS9114_RS01005	Inositol 2-dehydrogenase	2.42 ± 0.21	2.42 ± 1.36	–	–	–
	CGS9114_RS07530	Histidinol dehydrogenase	–	4.26 ± 2.09	–	–	8.14 ± 5.41
	CGS9114_RS05180	Shikimate 5-dehydrogenase	2.80 ± 1.42	–	–	–	–
	CGS9114_RS06205	Shikimate 5-dehydrogenase	–	2.62 ± 1.30	–	–	3.44 ± 1.87
	CGS9114_RS09125	Threonine dehydrogenase	2.35 ± 0.75	–	–	–	2.64 ± 0.83
	CGS9114_RS08410	3-Ketoacyl-ACP reductase	4.84 ± 0.76	5.05 ± 0.38	–	–	–
	CGS9114_RS09725	Short-chain dehydrogenase	–	–	–	2.92 ± 0.76	8.02 ± 1.98
	CGS9114_RS11770	Short-chain dehydrogenase	–	–	–	2.46 ± 0.25	–
	CGS9114_RS10460	Short-chain dehydrogenase	–	–	–	2.23 ± 0.78	–
	CGS9114_RS07680	Short-chain dehydrogenase	–	–	2.42 ± 1.10	2.25 ± 0.91	2.25 ± 0.98
	CGS9114_RS05810	Short-chain dehydrogenase	–	–	–	–	7.84 ± 6.49
	CGS9114_RS09230	6-Phosphogluconate dehydrogenase	–	–	–	3.13 ± 1.70	3.49 ± 2.63
ALDH	CGS9114_RS08415	Aldehyde dehydrogenase	8.22 ± 3.19	4.22 ± 0.51	–	–	–
	CGS9114_RS00305	Aldehyde dehydrogenase	5.80 ± 2.62	2.51 ± 0.79	–	–	–
	CGS9114_RS12030	Aldehyde dehydrogenase	5.56 ± 1.07	3.40 ± 0.32	–	–	–
	CGS9114_RS01955	Aldehyde dehydrogenase	4.60 ± 1.44	3.19 ± 0.35	3.75 ± 0.25	–	–
	CGS9114_RS10695	Aldehyde dehydrogenase	4.38 ± 2.50	–	–	–	–
	CGS9114_RS00855	Aldehyde dehydrogenase	2.88 ± 0.85	–	–	–	–
	CGS9114_RS04340	Aldehyde dehydrogenase	2.60 ± 0.70	2.41 ± 1.11	28.52 ± 6.52	–	–
	CGS9114_RS02715	Succinate-semialdehyde dehydrogenase	6.91 ± 1.72	5.32 ± 1.78	–	–	–
	CGS9114_RS10395	Methylmalonate-semialdehyde dehydrogenase	5.45 ± 1.19	2.25 ± 1.10	–	–	–
	CGS9114_RS09335	Glyceraldehyde-3-phosphate dehydrogenase	3.72 ± 0.63	–	–	2.32 ± 1.60	2.20 ± 0.62
Other oxidoreductase	CGS9114_RS04585	Heme peroxidase superfamily protein	5.01 ± 1.46	3.52 ± 1.09			
	CGS9114_RS01015	FAD-binding dehydrogenase	2.26 ± 0.11	–	–	–	–
	CGS9114_RS10360	Dehydrogenase	2.15 ± 0.74	–	–	–	–
	CGS9114_RS01830	Oxidoreductase	3.19 ± 1.20	2.07 ± 0.72	–	–	4.79 ± 2.36
	CGS9114_RS08040	Oxidoreductase	2.89 ± 0.64	4.28 ± 0.30	–	–	–
	CGS9114_RS02640	FAD-dependent oxidoreductase	3.07 ± 1.22	3.08 ± 1.16	–	–	–
	CGS9114_RS08560	Flavin oxidoreductase	3.83 ± 1.71	4.01 ± 1.41	–	–	–
	CGS9114_RS12040	FAD-dependent oxidoreductase	7.12 ± 2.49	3.51 ± 0.38	–	–	–

Table 1 (continued)

Genes	Genes ID	Functional annotation	Fold change				
			Furfural	HMF	HBA	Vanillin	Syringaldehyde
	CGS9114_RS01385	Oxidoreductase	2.38 ± 1.10	–	–	–	–
	CGS9114_RS03915	Oxidoreductase	2.36 ± 0.98	–	–	–	–
	CGS9114_RS09375	Multicopper oxidase	–	–	3.18 ± 0.28	9.42 ± 3.22	4.51 ± 1.62
	CGS9114_RS06005	Hypothetical protein	–	–	–	5.48 ± 3.20	4.01 ± 1.47
	CGS9114_RS01515	FAD-linked oxidoreductase	–	–	–	2.80 ± 0.61	2.87 ± 0.82
	CGS9114_RS11565	Oxidoreductase	–	–	–	4.78 ± 1.26	6.38 ± 3.27

– Relative expression means not up-regulated or up-regulated to relative lower level (less than twofold)

genes *CGS9114_RS00260* and *CGS9114_RS11970*, and one ALDH gene *CGS9114_RS00305* were up-regulated only under the stress of two furaldehydes. The gene, *CGS9114_RS09375*, encoding multicopper oxidase was significantly up-regulated against the three benzaldehydes, but not by the two furaldehydes. Protein homologous results using CDART (Conserved Domain Architecture Retrieval Tool: http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi) of NCBI showed that the protein domain architectures of the multicopper oxidase were homologous to CueO in *E. coli* which related to laccase (benzenediol:oxygen oxidoreductase) and L-ascorbate oxidase [26]. Laccase can oxidize vanillin, syringaldehyde, and 4-hydroxybenzoic acid with free oxygen as the acceptor in *Daedalea quercina*, *Trametes villosa*, *Trametes hirsute,* or *Perenniporia tephropora* [1, 3, 25, 42]. Thus, the result indicates that the conversion of benzaldehydes is related to the direct oxidation by oxygen (aerobic conversion). Utilizing the multicopper oxidase protein with oxygen as the substrate could instead of the NAD(P)-dependent oxidoreductases and reduce the redox power's consumption of the bacteria, then promote the oxidation of corresponding alcohols of benzaldehydes into its aldehyde and acid forms under aerobic condition. On the other hand, the oxidoreductase genes with strong response to furaldehydes and to benzaldehydes also showed some overlaps: total 11 genes including *CGS9114_RS01115*, *CGS9114_RS01975*, *CGS9114_RS10340*, *CGS9114_RS04000*, *CGS9114_RS07530*, *CGS9114_RS06205*, *CGS9114_RS09125*, *CGS9114_RS01955*, *CGS9114_RS04340*, *CGS9114_RS09335*, and *CGS9114_RS01830* were up-regulated expression against at least one furan aldehyde and one phenolic aldehyde. Specifically, one oxidoreductase gene *CGS9114_RS01115* was found to be up-regulated in response to all the five aldehyde inhibitors stress, with 5.54-, 4.30-, 2.25-, 2.69-, and 2.96-fold changes against furfural, HMF, HBA, vanillin, and syringaldehyde, respectively. The amino acid of the enzyme encoded by *CGS9114_RS01115* was recognized by CDART that contains the Zn_ADH7 domain which belonging to the medium chain reductase/dehydrogenase (MDR) family. The Zn-dependent alcohol dehydrogenase possessed the dual functions of reduction of aldehydes and oxidation of corresponding alcohols, which had been reported in various microorganisms such as *A. resinae*, *Zymomonas mobilis,* and *Cupriavidus necator* [14, 36, 40]. Comprehensive analysis of the responding genes between furaldehydes and benzaldehydes will contributes to understanding the biotransformation pathways information of the typical aldehyde inhibitors.

Biotransformation pathways of furaldehydes and benzaldehydes in *C. glutamicum*

The biotransformation pathways of the furaldehydes and benzaldehydes by *C. glutamicum* S9114 at molecular level were suggested according to the inhibitor biotransformation performance, transcriptional analysis, and homologous blasting results, as shown in Fig. 4. *C. glutamicum* S9114 reduced the aldehydes into corresponding alcohols by NAD(P)H-dependent ADH firstly, and then oxidized to their corresponding acids by NAD(P)-dependent ADH, NAD(P)-dependent ALDH or oxidase through the formation of aldehyde intermediates at low concentration levels. The three consequent conversion reactions composed the essential steps of furan aldehydes and benzaldehydes transformation in *C. glutamicum* S9114. The aldehyde group is believed to be the toxic component, instead of furan ring, aromatic groups or phenolic hydroxyl groups of the aldehyde inhibitors [16, 17, 40]. The important step for elimination toxicity of the five typical aldehyde inhibitors all followed the similar way by reducing or oxidizing of aldehyde group, rather than ring opening of furans or aromatics. The generation rate of five corresponding alcohols was faster than corresponding acids, indicating that the ADH or other NAD(P)H-dependent oxidoreductase genes took the important roles in reducing toxicity of aldehydes by reduction of aldehydes into less toxic alcohols.

Only the most significantly up-regulated genes are illustrated in Fig. 4 by selecting the genes with threshold of fold change greater than five for the two furaldehyde inhibitors and greater than three for the three benzaldehyde

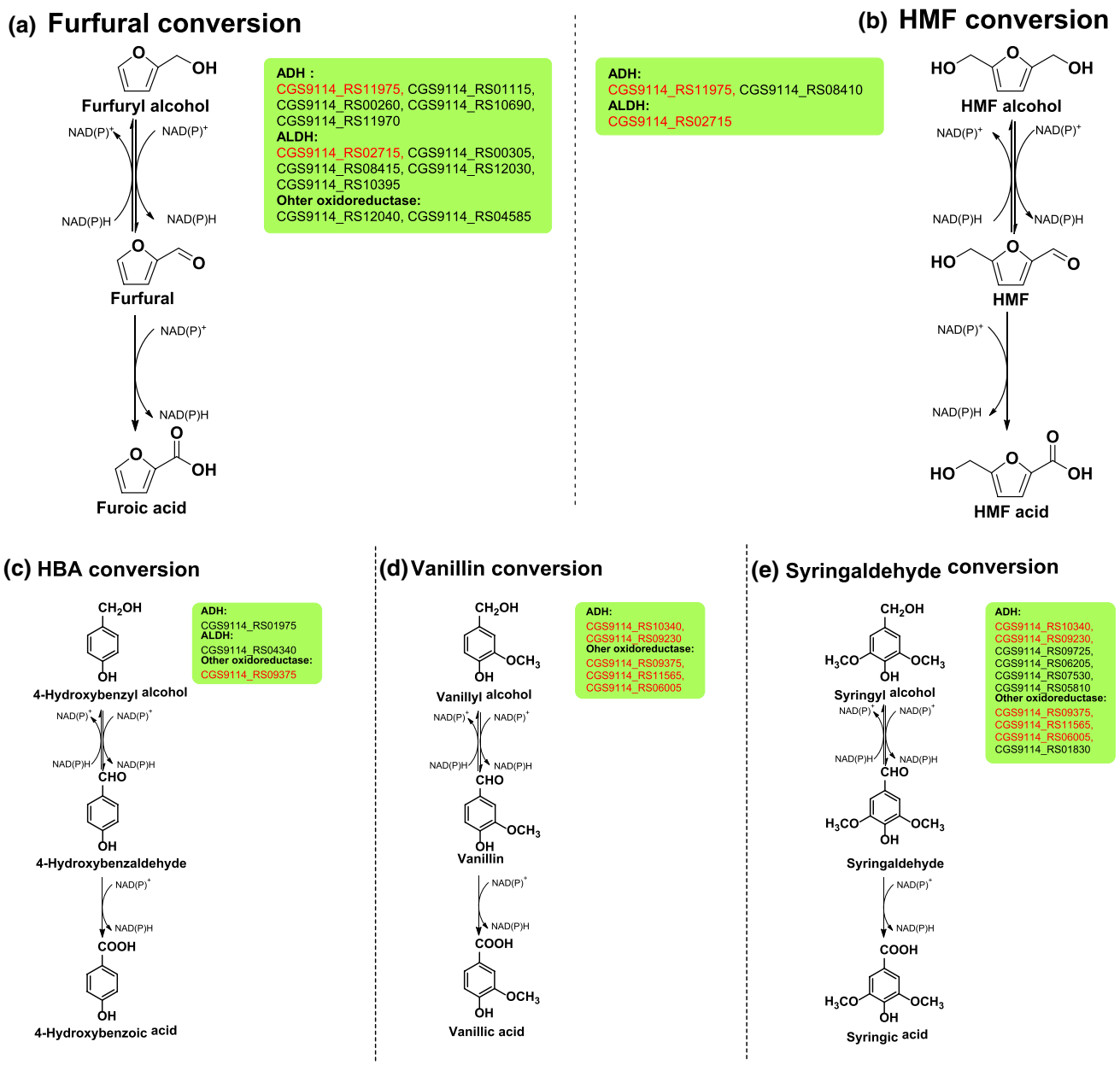

Fig. 4 Biotransformation pathways of furaldehydes and benzaldehydes by *C. glutamicum* S9114. **a** Furfural, **b** HMF, **c** HBA, **d** vanillin, **e** syringaldehyde. The most significant up-regulated genes were listed with a selection threshold of fold change ≥5.0 by furaldehydes or ≥3.0 by benzaldehydes. Genes in red color, the significant up-regulated genes shared by two furaldehydes or at least two benzaldehydes; genes in black color, the significant up-regulated genes only by the corresponding aldehyde

inhibitors. For the two furaldehydes, *CGS9114_RS11975* encoding inositol 2-dehydrogenase and *CGS9114_RS02715* encoding succinate-semialdehyde dehydrogenase showed significant up-regulation under both furfural and HMF stress. For the three benzaldehydes, two ADH genes including *CGS9114_RS10340* encoding inositol 2-dehydrogenase gene and *CGS9114_RS09230* encoding 6-phosphogluconate dehydrogenase gene, two other oxidoreductase genes *CGS9114_RS11565, CGS9114_RS06005* and one gene *CGS9114_RS09375* encoding multicopper oxidase showed significant up-regulation under at least two benzaldehydes stress. These explored valuable genes against treatment of several kinds of aldehydes in *C. glutamicum* might take a more important role in the biotransformation pathways of aldehyde inhibitors; thus, higher expression of the enzymes is examined for improving the furaldehydes and benzaldehydes biotransformation rate.

Expressing the genes with strong response to inhibitors in *C. glutamicum*

The genes with significant response to furaldehydes or benzaldehydes at transcriptional level were separately inserted into an expression plasmid pEFTUmob and then transformed into *C. glutamicum* S9114. The selection of the genes for overexpression was based on the transcription levels of the genes with a fold change greater than 5.0 for furaldehydes, greater than 3.0 for benzaldehydes or the genes significantly in response to multiple inhibitors such as *CGS9114_RS01115* and *CGS9114_RS04340*. For the conversion evaluation of furaldehydes, the selection genes included *CGS9114_RS01115*, *CGS9114_RS04340*, *CGS9114_RS00260*, *CGS9114_RS11975*, *CGS9114_RS10340*, and *CGS9114_RS02715*. For the conversion evaluation of benzaldehydes, the selection genes included *CGS9114_RS01115*, *CGS9114_RS09375*, *CGS9114_RS09725*, *CGS9114_RS04340*, *CGS9114_RS10340*, *CGS9114_RS09230*, *CGS9114_RS11565*, and *CGS9114_RS06005*. The cell growth and inhibitors' conversion ratio of the recombinants were evaluated in synthetic medium added with proper concentration of furfural, HMF, HBA, vanillin, or syringaldehyde, respectively (Fig. 5). Figure 5a, b shows that the cell growth and furaldehydes' conversion ratio were increased by all the six recombinants comparing to control. The maximum increases of furfural and HMF conversion ratio appeared when the gene *CGS9114_RS11975* was expressed (10.64% for furfural and 9.29% for HMF). On the other hand, the expression of the genes with strong response to benzaldehydes facilitated the cell growth to a certain extent, but the improvement of benzaldehydes conversion

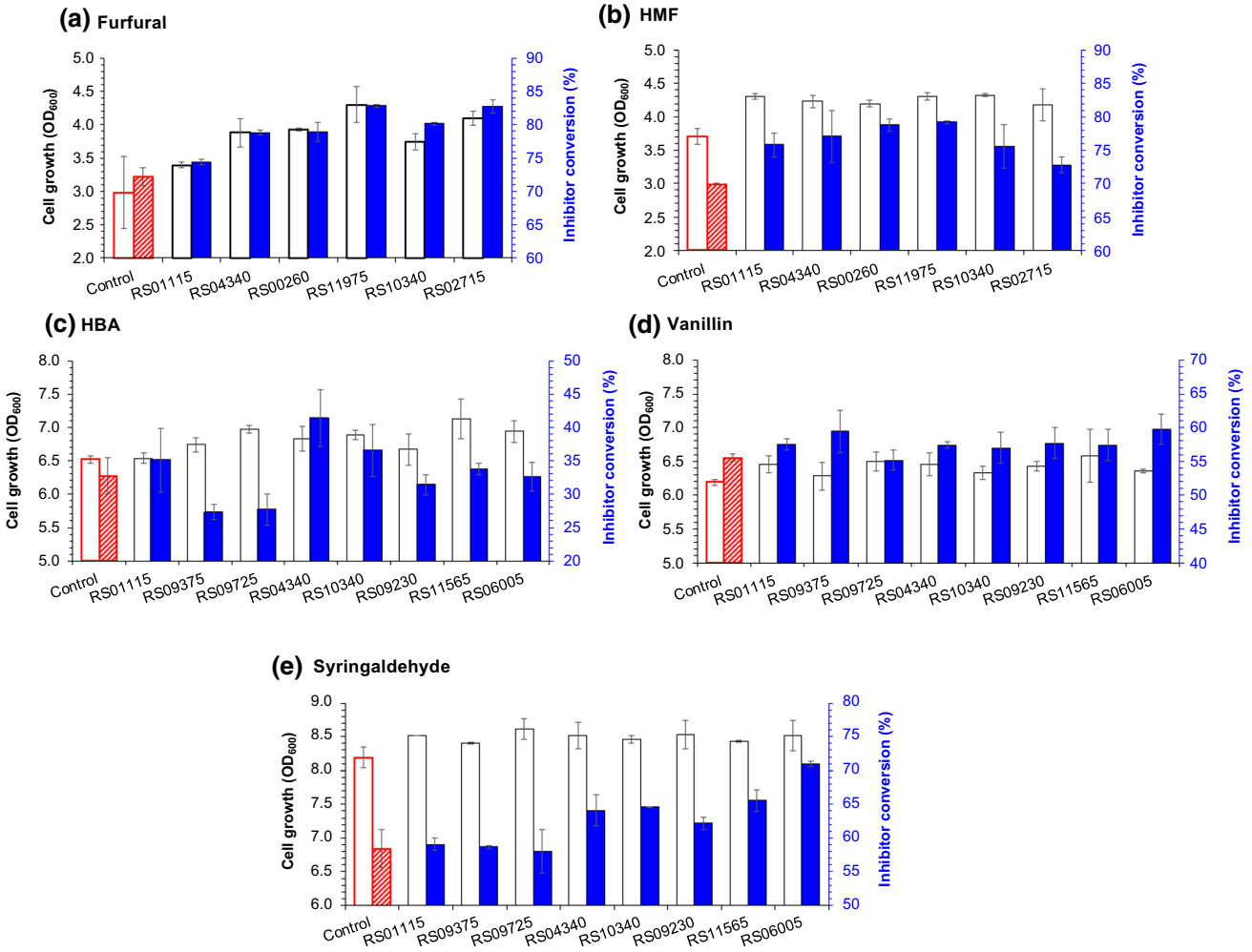

Fig. 5 Cell growth and inhibitors conversion ratio of recombinant *C. glutamicum* S9114. **a** Furfural, 17 mM, **b** HMF, 17 mM, **c** HBA, 6.6 mM, **d** vanillin, 6.6 mM, **e** syringaldehyde, 0.8 mM. Each gene was separately inserted into an expression plasmid pEFTUmob and then transformed into *C. glutamicum* S9114. Control: only transformed the plasmid pEFTUmob into *C. glutamicum* S9114. Conditions: pH 7.0, 30 °C and 200 rpm in synthetic medium for 8 h, 8 h, 16 h, 16 h, and 12 h by furfural, HMF, HBA, vanillin, and syringaldehyde, respectively. Mean values were presented with error bars representing at least two standard deviations

ratio was not as significant as that of furaldehydes (Fig. 5c, e). Among the changes of benzaldehydes' conversion by the recombinants, the expression of *CGS9114_RS04340* improved the conversion ratio of HBA by 8.67%; the expression of *CGS9114_RS06005* improved the conversion ratio of vanillin and syringaldehyde by 4.25% and 12.52%, respectively. Five aldehydes conversion ratio were all improved by the expression of ADH gene *CGS9114_RS01115*, which was up-regulated under the stress of all five aldehyde inhibitors.

These results showed that overexpression of the related genes improved both the cell growth and the aldehydes conversion ratio, while the improvement range was limited. Bioconversion of the aldehydes is a complicated and synergistic result of enzymes encoded by aforementioned multiple genes. Deletion or complementation of a single gene may not lead to a significant change on bioconversion performance of specific inhibitors. Perhaps, a stepwise and systematic modification on the complete inhibitor biotransformation pathway and the related transport/regulation should be performed, instead of the functional verification of a single gene. Complete elucidation of the tolerance mechanisms to various inhibitors in *C. glutamicum* is very important and also a long-term target requires a systematic work on the multiple inhibitor biotransformation pathways. The transcriptional analysis is the first step towards this long-term goal by revealing the potential key genes on the inhibitor conversions, which will provide a rich gene library for future metabolic modification of general biorefinery strains. We will continue multiple gene deletion or complementation experiments in our future work.

Conclusions

Tolerance and biotransformation analyses of *C. glutamicum* S9114 for the five lignocellulose-derived furaldehyde and benzaldehyde inhibitors were comprehensively analyzed. Multiple oxidoreductase genes responsible for aldehyde inhibitors conversion to the corresponding alcohols and acids were identified by qRT-PCR. The gene expression responses to furaldehyde and benzaldehyde inhibitors showed significant difference. Genes involved in biotransformation pathways of five aldehyde inhibitory compounds were finally suggested based on the inhibitor conversion performance, genome annotation, and the transcriptional analysis. One gene *CGS9114_RS01115* encoding alcohol dehydrogenase was up-regulated in response to all five aldehyde inhibitors and the overexpression of this gene in *C. glutamicum* improved the conversion ratio of five aldehydes to a certain extent. This study could provide valuable genetic resources for inhibitor conversion and facilitate the development of robust industrial fermenting strain in future lignocellulose biorefineries.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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