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# Simultaneous enhancement of lignin-derived inhibitor tolerance and lipid accumulation of oleaginous yeast *Trichosporon cutaneum* by adaptive evolution

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ABSTRACT

Inhibitory compounds should be removed from the pretreated lignocellulose feedstock before fermentative production of microbial lipid by oleaginous yeast. Biodetoxification effectively degrades furan aldehydes and weak organic acids, but is less efficient against complex and various lignin-derived phenolic aldehydes. Residual phenolics in lignocellulose hydrolysates require a tolerant fermentation strain. This study adopted the long-term adaptive evolution combined with centrifugal enrichment in corn stover hydrolysate to simultaneously improve the phenolic inhibitors tolerance and lipid synthesis of *Trichosporon cutaneum*. The obtained mutant *T. cutaneum* MS28 showed significant improved performances of phenolic aldehydes tolerance and lipid accumulation. The final cellulosic lipid production of *T. cutaneum* MS28 utilizing corn stover reached 33.8 ± 0.1 g/L, approximately 6-fold higher than that of the starting strain. Transcriptional analysis suggested that *T. cutaneum* MS28 had more active lignocellulose-derived sugars metabolism and lipid synthesis shunts. The intracellular contents of lipid synthesis precursors including NADPH and acetyl-CoA increased 1.7–3.0 folds. This adaptive evolution in lignocellulose hydrolysate combined with centrifugal enrichment is an efficient tool to targeted enhancement of inhibitors tolerance and cellulosic lipid production of oleaginous yeast.

## 1. Introduction

Microbial lipid produced by oleaginous yeast have been considered as an intriguing alternative for the biodiesels and oleochemical industrials [13,2,34,5]. Over 70% of the total costs of microbial lipid production and its utilization as feedstock for biodiesel production is related to the raw materials and substrate [8,37]. To reduce the raw feedstock costs, the microbial lipid production from cheap lignocellulose biomass by biorefining process had been widely investigated [32,37,8]. Pretreatment is the essential step in overcoming the recalcitrance of lignocellulose feedstock to release fermentable sugars [23]. However, during the pretreatment operation, toxic compounds such as furan aldehydes, organic acids, and phenolic aldehydes are generated from the over-degradation of cellulose, hemi-cellulose, and lignin. These toxic compounds showed strong inhibitory effect on the oleaginous yeasts in the downstream fermentation step and should be removed completely before lipid fermentation [3,26].

Various chemical and physical detoxification methods had been employed for the elimination of the toxic compounds from the pretreated biomass. However, most of these methods possess several disadvantages such as fermentable sugars loss, wastewater generation, high energy input [16,31]. Biological detoxification (Biodetoxification) using special microorganisms is considered as one of the most proper approaches. Biodetoxification using the unique fungi *Amorphotheca resinae* ZN1 or *Paecilomyces variotii* FN89 enables the efficient removal of 5-hydroxymethyfurfural (HMF), furfural, and acetic acid with negligible loss (less than 2%) of fermentable sugars [18,45]. However, for the degradation of lignin derived phenolic aldehydes represented by 4-hydroxybenzaldehyde (4-HBA), vanillin and syringaldehyde, the biodetoxification rate is slow due to their wide variety and a prolonged biodetoxification period is required [46]. The prolonged operation inevitably caused an extra loss of free sugars [19,45].

Metabolic engineering and adaptive laboratory evolution are common and efficient tools to improve the inhibitors tolerance of oleaginous yeast [27]. Although tremendous progress in the lipid metabolic engineering has been realized in the last decade [4], the rational metabolic

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engineering was often difficult to balance the cell growth, lipid biosynthesis, and inhibitors tolerance [35]. Comparing with the rational metabolic engineering, the adaptive evolution is easier to perform by culturing over multiple generations under a specific selection pressure. The satisfying phenotypes are maintained, while the undesirable phenotypes are gradually phased out. The adaptively evolved cells can reconfigure their metabolism to reinforce the improved phenotype [36]. The adaptive evolution in inhibitors-contained medium has been developed to improve the tolerance of *Rhodosporidium toruloides* or *Yarrowia lipolytica* to phenolic aldehyde inhibitors [27,50]. Nevertheless, in these reported adaptive evolution cases, the improvement of inhibitors tolerance and the enhancement of lipid accumulation are usually not synergistic. It is promising to simultaneously improve both the inhibitors tolerance and lipid production of oleaginous yeast by introducing an efficient screening method for isolating high lipid content mutant cells in addition to the adaptive evolution in inhibitors-contained medium.

*Trichosporon cutaneum* with a wide range of substrates spectrum can utilize lignocellulose-derived carbohydrates to accumulate lipid [43]. In this research, the adaptive evolution of *T. cutaneum* was conducted in corn stover hydrolysate containing low levels of phenolic aldehydes aiming to improve its inhibitors tolerance. The adaptive evolution was combined with an efficient screening method, centrifugal enrichment method, to simultaneously enhance the lipid biosynthesis. The transcriptional analysis was further performed to attempt to reveal the potential genes responsible for the enhanced inhibitors tolerance and lipid synthesis in the finally obtained strain.

## 2. Materials and methods

### 2.1. Strains

*T. cutaneum* ACCC 20271 was purchased and used as the parental strain for adaptive evolution. *Paecilomyces variotii* FN89 was isolated in our previous study [45], which was used for biodetoxification.

### 2.2. Reagents

Cellulase was purchased from Novozymes (Beijing, China, Cellic CTec 2.0). Vanillin, syringaldehyde, 4-HBA, glucose, and other reagents were purchased from Chinese Sinopharm Chemical Reagent.

### 2.3. Pretreated biomass preparation

The main compositions of raw corn stover includes 33.9 ± 0.3% (w/w) of cellulose, 17.8 ± 0.2% (w/w) of xylan and 9.2 ± 0.1% (w/w) of ash (Sluiter et al., 2012). The milled corn stover were about 10 mm in length, and then used for dry acid pretreatment according to our previous method [24].

### 2.4. Corn stover hydrolysate preparation

The mild solid biodetoxification was carried out on pretreated corn stover by inoculating the fungus *P. variotii* FN89 [45]. The inhibitors including acetic acid, furfural, and HMF were quickly removed within 36 h during the biodetoxification period, however approximately 20% of the phenolic aldehydes were left, represented by 4-HBA, vanillin, and syringaldehyde [46]. Then the pretreated corn stover with residual phenolics was hydrolyzed by adding 5.5 mg/g cellulolytic protein at ~20% (w/w) solids loading, 50 ℃ for 48 h [39]. The obtained slurry was centrifuged and filtered to collect clarified supernatant. The fermentation nutrients including 1.0 g/L $KH_2PO_4$, 1.0 g/L yeast extract, 0.5 g/L $MgSO_4.7\ H_2O$, and 0.22 g/L $(NH_4)_2SO_4$ were added. The clarified hydrolysate containing 80.1 g/L glucose, 21.2 g/L xylose, 0.8 g/L galactose and 2.0 g/L mannose and arabinose, 0.08 g/L residual 4-HBA, 0.13 g/L residual vanillin, and 0.07 g/L residual syringaldehyde.

### 2.5. Adaptive evolution with centrifugal enrichment

The adaptive evolution was performed in 500 mL flask containing 50 mL of clarified hydrolysate. The conditions for cultivation were 180 rpm, 30 ℃, 120 h. Then 20 mL of the broth was centrifugated for 3 min using Bechman Coulter Avanti J-26 XP. The centrifugal force gradually increased from 1000 g to 12,000 g during 70 times of transfer**.** The cells containing higher lipid showed lighter density [17], thus the higher lipid content cells would float on the broth after centrifugal enrichment. 5 mL of the upper broth containing lighter cells was pipetted as the seed for the next round cultivation. The final obtained cell pellets were transferred in YPD medium and a single colony on the petri dish gel was finally isolated. The isolate showed the phenotypic stability and was named as *T. cutaneum* MS28. For inhibitors tolerance evaluation of *T. cutaneum* on synthetic medium plate, the seed was cultured for 24 h, and then diluted to OD600 of 1.0. The seed culture was subsequently diluted 0×, 10×, 100×, and 1000× in gradient. 4 μL of the diluted seed culture was pipetted and added dropwise onto the synthetic medium plates containing different phenolic aldehydes.

### 2.6. Cell morphology, and measurement of cell diameter and density

The preparation of the observations by optical microscope (OM) and transmission electron microscope (TEM) were performed as previously described [25]. The photographs of OM were caught using Olympus HT2000CN (Japan). The photographs of TEM were caught using JEM-1400 Flash (JEOL, Japan).

The viscosity of fermentation broth was measured at shear rate from 4.4 to 52.2 $s^{-1}$ at 25 °C on Brookfield DV2T viscometer (Stoughton, Middleboro, MA, USA).

Cell diameter was analyzed using Nano Measurer software (version 1.2). The density of fermentation broth and supernatant were measured on Density Meter DMA 4500 M (Anton Paar, Graz, Austria). The wet cell density was measured by harvesting the cells in fermentation broth and centrifuged at 12,000 g for 30 min to precipitate all suspended cells [7,12]. The density of wet cell mass ($\rho_w$, kg/$m^3$) was calculated according to Eq. (1):

$$w/\rho_b = w_1/\rho_w + (w - w_1)/\rho_s \quad (1)$$

where $\rho_b$, the density of fermentation broth (kg/$m^3$); $\rho_s$, the density of supernatant (kg/$m^3$); $w$ was the weight of fermentation broth (kg); $w_1$, the weight of wet cells (kg). After centrifugation, the cells were assumed to be close-packed spheres. The volume ratio of the close-packed cells to the wet cells containing liquid was assumed as 0.74 as previously described [6]. The cell density ($\rho_c$, kg/$m^3$) was calculated according on Eq. (2):

$$\rho_c \times 0.74 + \rho_s \times 0.26 = \rho_w \times 1 \quad (2)$$

### 2.7. Cellulosic lipid fermentation

Simultaneous saccharification and co-fermentation (SSCF) was applied to evaluate the cellulosic lipid accumulation capacity of *T. cutaneum* strains. The partially biodetoxified corn stover was prehydrolyzed by adding 4 mg cellulolytic protein/g dry substrate at 30% (w/w) solids loading, 150 rpm for 12 h. Then the *T. cutaneum* seed was inoculated at 10% (w/w) inoculum ratio. The SSCF was performed at 30 ℃, 1.0 vvm, pH 5.0 for 72 h.

### 2.8. RT-qPCR

The two *T. cutaneum* strains were cultured at 30 ℃ in corn stover hydrolysate for 24 h. The cells were collected. RNA extraction and quantification, reverse transcription reactions, and RT-qPCR were carried out by using the previous methods [44]. The GenBank ID of the genome of *T. cutaneum* ACCC 2027 was ID LTAL00000000 [38].

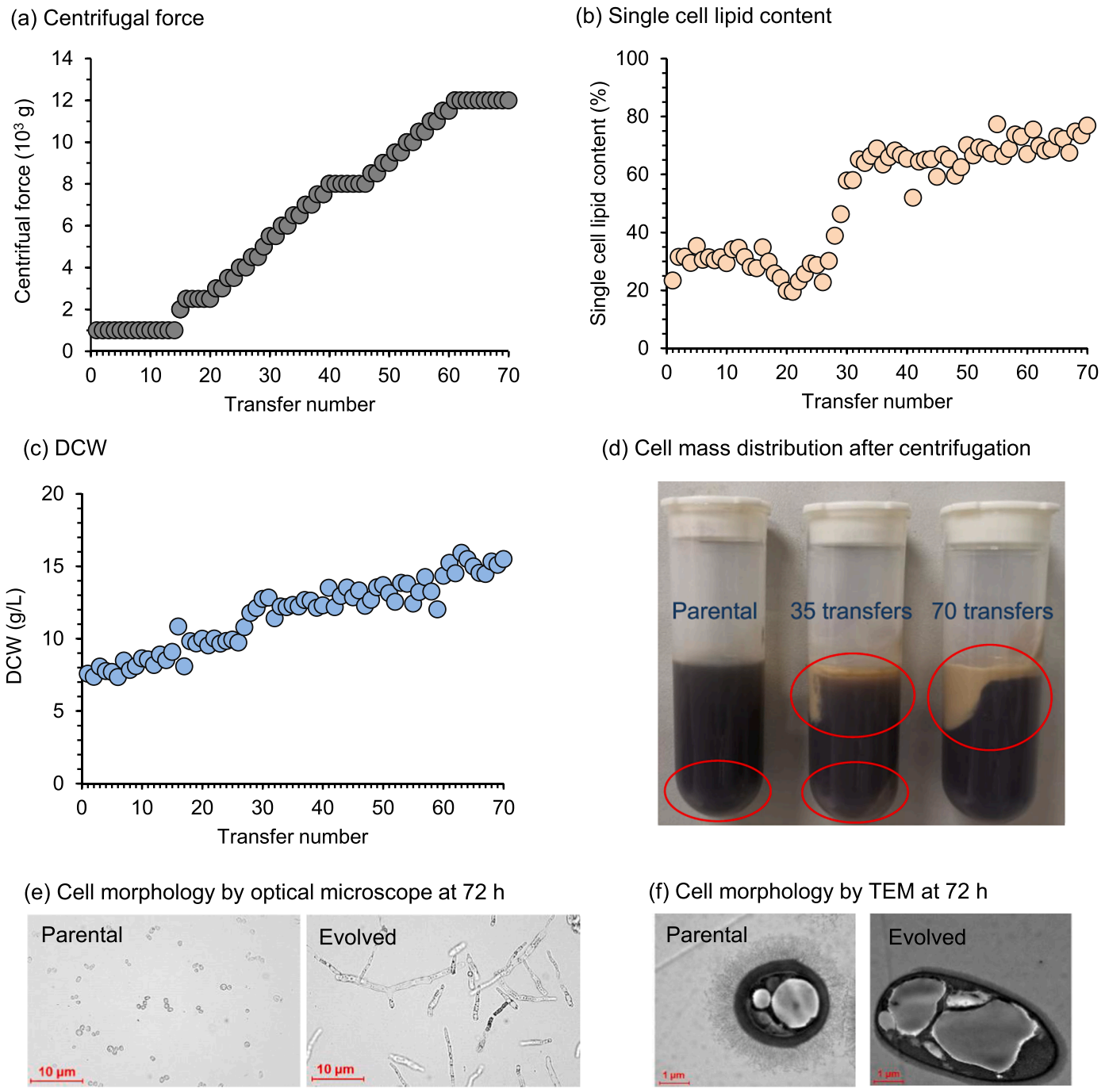


**Fig. 1.** Adaptive evolution of *T. cutaneum* in 20% (w/w) solids loading corn stover clarified hydrolysate combined with centrifugal enrichment. The changes of centrifugal force for screening high-lipid content cells during the adaptive evolution (a); the changes of single cell lipid content (b); the changes of dry cell mass (c); the cell mass distribution after centrifugation (d); the morphology of *T. cutaneum* in 20% (w/w) solids loading corn stover clarified hydrolysate by optical microscope (e); and TEM (f).

### 2.9. Analysis

Sugars, acetic acid, furfural, and phenolic aldehydes were analyzed by HPLC method [18,45]. In brief, glucose, xylose, acetic acid, furfural were analyzed by Shimadzu HPLC system equippen with HPX-87 H column (Bio-rad Aminex) and RID-10A detector [45,18]. Galactose, arabinose and mannose were analyzed by the refractive index detector (RID-10A) [25]. Vanillin, 4-HBA, syringaldehyde and their corresponding derivatives (acids and alcohols) were determined by YMC-Pack ODS-A column and UV/Vis deterctor (SPD-20A) [45]. Cell wall components including glucan, chitin, and mannan were determined according to the previous reports [9,30]. The intracellular acetyl-CoA and NADPH was measured using analytical kit (Solarbio Biotech, Beijing, China; Beyotime Biotech, Shanghai, China), respectively. Dry cell weight (DCW) was measured by oven drying method. The lipid was extracted by phenol-chloroform extraction [11].

### 2.10. Statistical analysis

Results are shown as mean ± standard error of the mean (SEM). The graphs were prepared using OriginPro 2021 version (OriginLab Corporated, Northampton, Massachusetts, USA) and Office Excel. Variations

**Table 1**
Cell parameters of *T. cutaneum* MS28 compared to parental strain in hydrolysate.

| | Parental | *T. cutaneum* MS28 |
|---|---|---|
| Cell radius (m) | $2.85 \times 10^{-6}$ | $6.93 \times 10^{-6}$ |
| Cell volume ($m^3$) | $9.70 \times 10^{-17}$ | $1.39 \times 10^{-15}$ (~14 folds) |
| Cell density ($kg/m^3$) | 1156.3 | 992.1 (lower than broth)[a] |

[a] The density of clarfied hydrolysate was 1025.6 $kg/m^3$.

were considered statistically significant at * $P < 0.05$, * *$P < 0.01$), and * **$P < 0.001$.

## 3. Results and discussion

### 3.1. Adaptive evolution combined with centrifugal enrichment in *T. cutaneum*

The overall adaptive evolution was performed for 350 days' (70 transfers) and the centrifugal force increased from 1000 g to 12,000 g (Fig. 1a). The lipid content of *T. cutaneum* was elevated from the initial 23.3% (at the first inoculation) to the ending 76.8% (at the 70th transfer) (Fig. 1b), and the dry cell weight increased from 7.6 g/L to 15.5 g/L (Fig. 1c). The higher lipid content of evolved cells led to the lighter cell density, which was even below the density of the clarified corn stover hydrolysate (Table 1). After the centrifugation, more and more cell masses were distributed in the up layer of the broth as the transfer proceeded (Fig. 1d).

Meanwhile, the long-term evolution changed the morphology of *T. cutaneum*. The optical microscope observation showed that *T. cutaneum* MS28 was in the form of large ellipsoid shape, while the parental cells were in the small spherical shape. The average cell volume of *T. cutaneum* MS28 was ~14-fold bigger than that of the parental strain ($1.39 \times 10^{-15}$ $m^3$ *vs.* $9.70 \times 10^{-17}$ $m^3$) (Table 1). The TEM images showed that the lipid bodies occupied almost the entire intracellular space of *T. cutaneum* MS28, and the cell wall was much thinner than that of the parental strain .

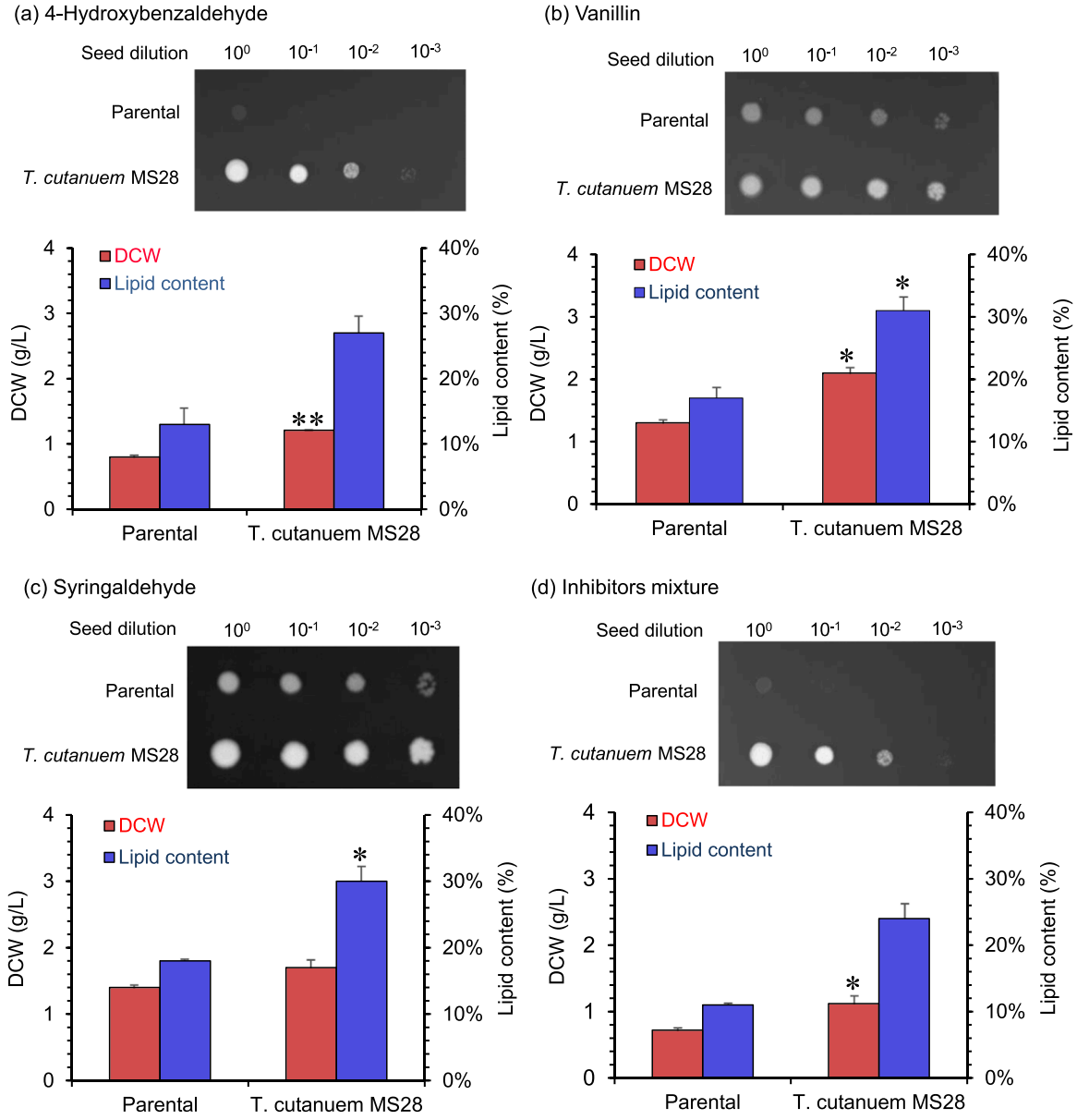


**Fig. 2.** Phenolic aldehyde inhibitors tolerance of *T.cutanuem* MS28 in synthetic medium. The cell viability of parental strain and *T.cutanuem* MS28 were observed by culturing on synthetic medium plate containing 0.6 g/L 4-HBA (a), 0.5 g/L vanillin (b), 0.5 g/L syringaldehyde (c), or above inhibitors mixture (d) at 30 ℃ for 48 h. Dry cell weight and single cell lipid content were measured after 120 h of flask fermentation.

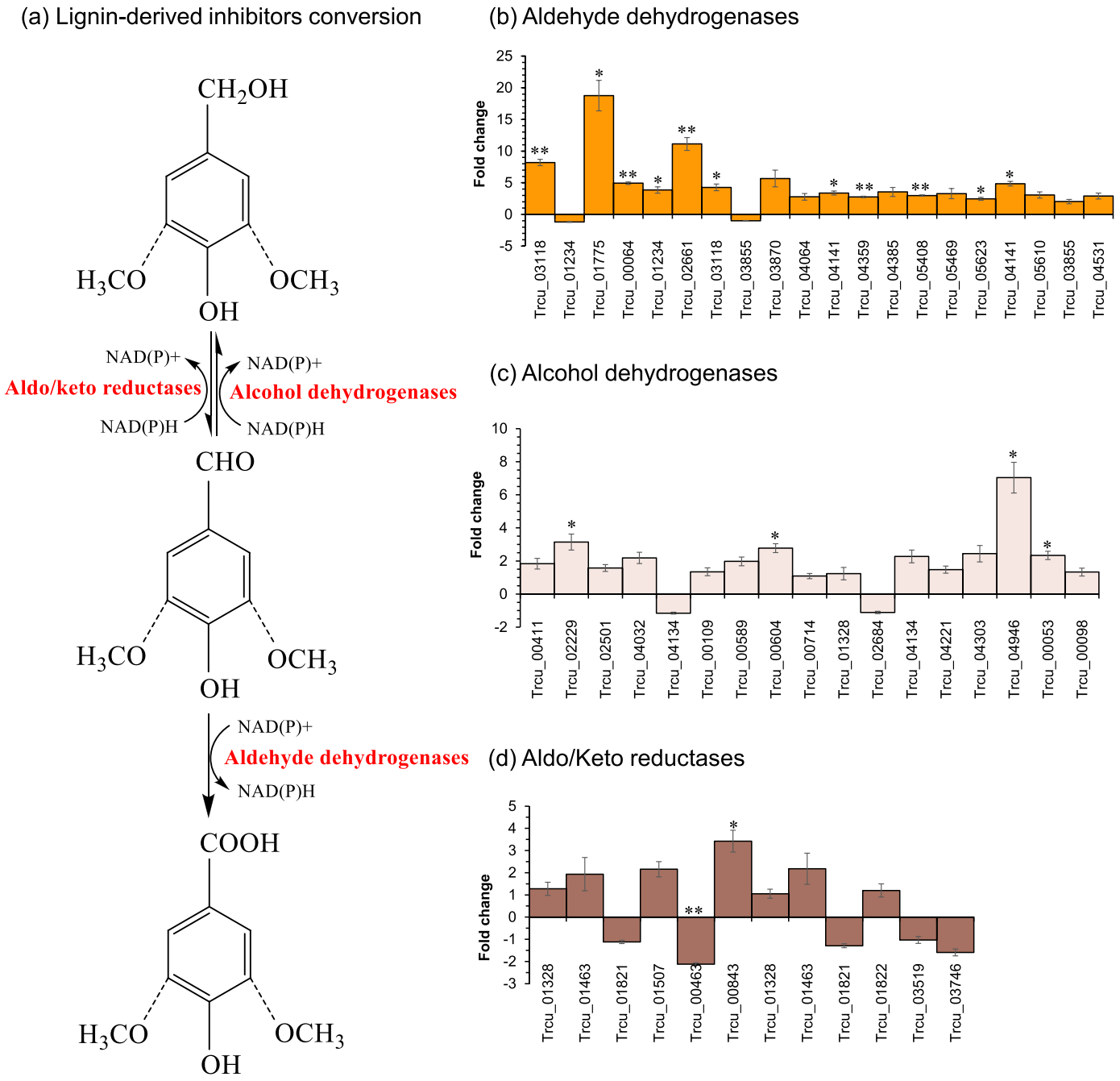


**Fig. 3.** Transcriptional analysis of the genes responsible for phenolic aldehyde conversion in *T. cutaneum* MS28. The pathway of lignin-derived inhibitors conversion (a); the differential expressions of aldehyde dehydrogenases (b); the differential expressions of alcohol dehydrogenases (c); and the differential expressions of aldo/keto reductase (d). The samples were collected after cultured in 20% (w/w) solids loading corn stover clarified hydrolysate for 24 h. The gene expression levels were normalized against the parental strain *T. cutaneum* ACCC 20271. Each RT-qPCR was performed at least three times.

### *3.2. Evaluations of T. cutaneum MS28 in lignin-derived inhibitors tolerance*

The tolerance of obtained *T. cutaneum* MS28 strain to phenolic aldehyde inhibitors was evaluated in the medium containing inhibitors (Fig. 3). Among the three lignin-derived inhibitors, 4-HBA showed the strongest negative effects on cell growth and lipid accumulation of *T. cutaneum* (Fig. 3a). *T. cutaneum* MS28 has higher cell viability compared with the parental strain in the presence of three phenolic aldehydes. Both DCW and lipid content of *T. cutaneum* MS28 were higher than that of parental strain with the addition of single or mixed inhibitor either.

The improvement of the tolerance to phenolic aldehydes in *T. cutaneum* MS28 indicated the occurrence of the transcriptional changes in the genes related to phenolic aldehyde degradations. Zhang & Bao [48] reported that phenolic aldehydes were converted to the corresponding alcohols by alcohol dehydrogenase or aldehyde reductase in an irreversible reaction, then the aldehydes were oxidized to the corresponding phenolic acids by aldehyde hydrogenase at a low concentration in *T. cutaneum* (Fig. 4a). The transcriptional analysis was further conducted, and focused on the genes related to phenolic aldehyde conversion (Fig. 4b–d). The results suggested that the genes encoding aldehyde dehydrogenases were generally up-regulated more than 2-fold (except two slight down-regulated). Trcu_01775 gene

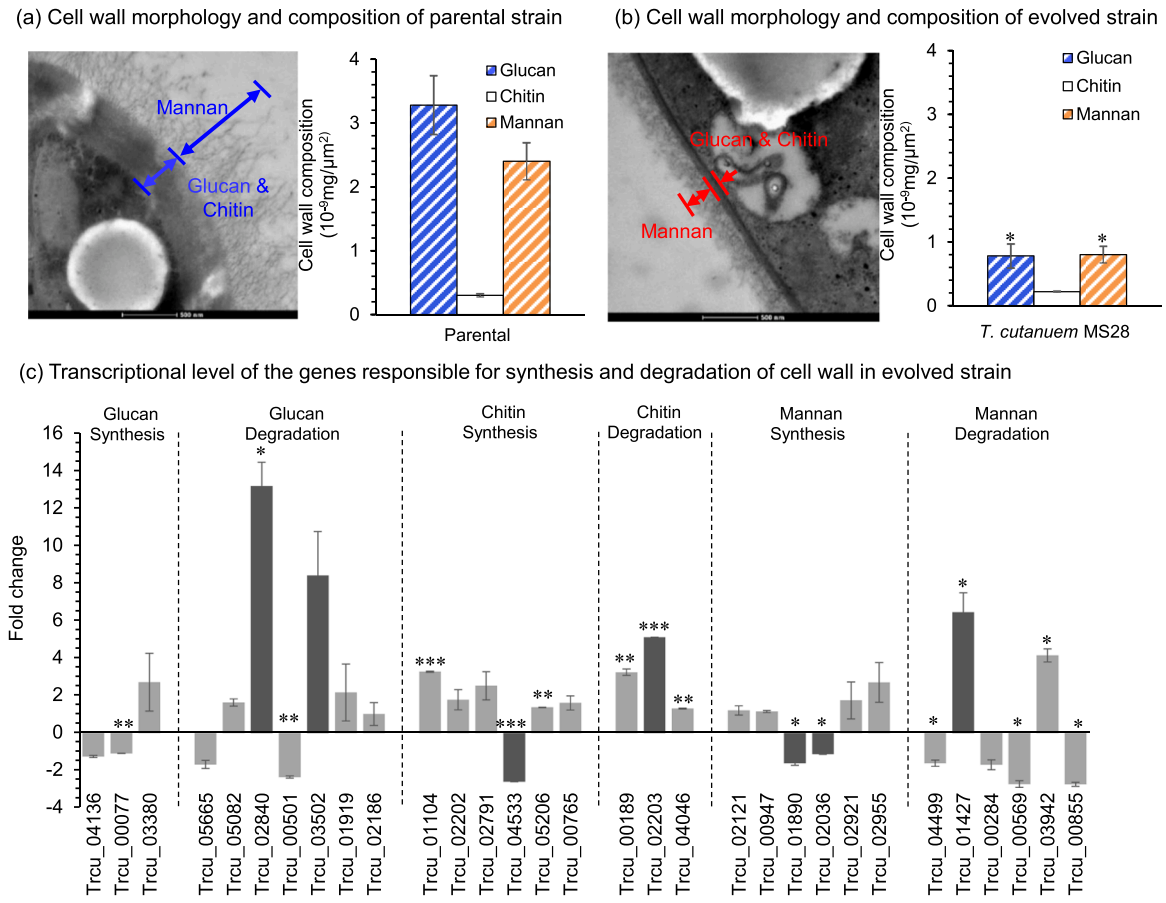


**Fig. 4.** Long-term adaptive evolution changed the cell wall characterizations. (a) Cell wall structure and compositions. The cell wall morphology of the parental strain was referenced from the previously preliminary study [25]. Copyright 2022 Wiley Periodicals LLC. (b) Transcriptional level changes of the genes related to cell wall component synthesis and degradation. The samples were collected after cultured in 20% (w/w) solids loading corn stover clarified hydrolysate for 24 h. The gene expression levels were normalized against the parental strain *T. cutaneum* ACCC 20271. Each RT-qPCR was performed at least three times.

encoding aldehyde dehydrogenase ($NAD(P)^+$) was significantly up-regulated 18.8-fold, which may play a vital role in phenolic aldehydes conversion.

### 3.3. Long-term adaptive evolution changed the cell wall compositions of *T. cutaneum*

The cell wall of yeast is mainly composed of β-glucan, mannan, chitin, and proteins [40]. The composition and structure of cell wall change dynamically as a response to the environmental stress [47]. Gu et al. (2019) found that after the adaptive evolution of *Saccharomyces cerevisiae* in the presence of multiple phenolic acids, the evolved yeast showed thicker cell wall and membrane and smaller cell size than those of the parental strain [15]. In this study, after the long-term adaptive evolution with centrifugal stress, the evolved cell showed significantly thinner mannan layer, glucan, and chitin layer by TEM observation (Fig. 4a and b). The content of glucan, chitin, and mannan was decreased by 76.2%, 26.7%, and 66.7%, respectively. For lipid recovery from oleaginous yeast by cell lysis, the main obstacle is the rigid cell wall due to the high mannan and chitin contents [22]. The lower chitin and mannan contents in evolved cells would facilitate the cell lysis and lipid extraction in industrial-scale production.

The transcriptional changes of the genes related to cell wall component synthesis and degradation were further determined by RT-qPCR (Fig. 4c). The results showed that three genes Trcu_02840, Trcu_03502, Trcu_02203, and Trcu_01427 encoding endo-1,3(4)-β-glucanase, endoglucanase C, chitinase, and 1,2-α-mannosidase related to glucan, chitin and mannan degradation were significantly up-regulated by 13.1-fold, 8.4-fold, 5.1-fold, and 6.5-fold. While the genes Trcu_04533, Trcu_01890, Trcu_02036 encoding chitin synthase, mannose-1-phosphate guanylytransferase, and mannosytransferase related to chitin and mannan synthesis were down-regulated by 2.6-fold, 1.6-fold, and 1.1-fold. The transcriptional changes of these key genes involved in the synthesis and degradation of cell wall components contributed to the remodelling of the cell wall structure in response to strong centrifugal force stress.

### 3.4. Evaluation of lipid fermentation of *T. cutaneum* MS28 using corn stover feedstock

The cellulosic lipid production of *T. cutaneum* MS28 was evaluated using corn stover feedstock and compared with that of the parental strain (Fig. 5). The cell growth was not measured due to the presence of lignin solids in broth. The attempts also have been made to determine the changes in the concentrations of phenolic aldehydes and theirs corresponding derivatives during the fermentation, but the accurate quantification was difficult due to their low levels and the complex compositions of the corn stover hydrolysate. Our previous study has suggested that *T. cutaneum* can utilize 4-HBA for lipid accumulation, while only convert syringaldehyde and vanillin to less toxic phenolic alcohols and acids owing to the space shelling of methoxyl group(s) in the chemical structures [20].

All fermentable sugars including glucose, xylose, mannose, arabinose, and galactose were consumed by *T. cutaneum* MS28 at 72 h during SSCF (Fig. 5a–d), and the cellulosic lipid titer reached 33.8 ± 0.1 g/L, approximately 6-fold higher than that of parental strain (5.9 ± 0.5 g/L)

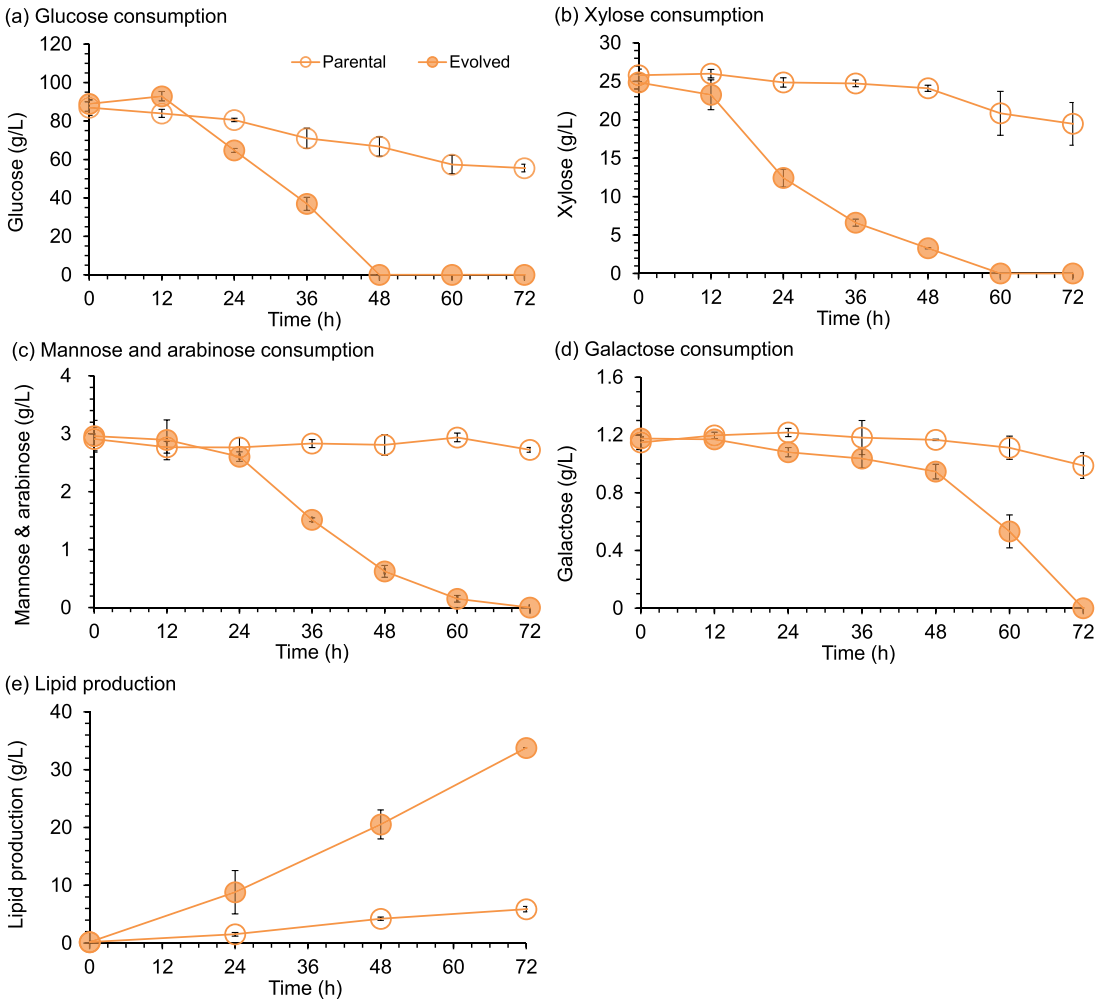


**Fig. 5.** Cellulosic lipid production by SSCF. (a) Glucose consumption; (b) xylose consumption; (c) mannose and arabinose consumption; (d) galactose consumption; and (e) lipid accumulation and cell growth. The SSCF was conducted using 30% (w/w) solids loading corn stover, 4 mg protein/g dry substrate, 30 °C, 180 rpm for 72 h.

**Table 2**
Cellulosic lipid production performances by batch fermentation.

| Strain | Feedstock | Titer (g/L) | Yield (g/g) | Productivity (g/L/d) | Refs |
|---|---|---|---|---|---|
| *Mortierella isabellina* DSM1414 | Corncob | 11.6 | 0.11 | 0.61 | [21] |
| *Schizochytrium* sp. HX-308 | Corn straw | 5.3 | 0.13 | 2.1 | [41] |
| *Lipomyces starkeyi* AS 2.1560 | Sigmacell cellulose | 9.5 | / | 2.4 | [14] |
| *Solicoccozyma terricola* DBVPG 5870 | Cardoon stalks | 13.8 | ~0.13 | 1.4 | [1] |
| *Lipomyces tetrasporus* Y-11562 | Corn stover | 29.0 | 0.15 | 8.1 | [33] |
| *Cutaneotrichosporon oleaginosum* ATCC 20509 | Corn stover | 14.4[a] | 0.24 | 3.6 | [42] |
| *Rhodosporidium toruloides* Y4 | Jerusalem artichoke | 17.2[a] | / | 9.1 | [49] |
| *T. cutaneum* ACCC 20271 | Corn stover | 5.9 | 0.05 | 2.0 | This study |
| *T. cutaneum MS28* | Corn stover | 33.8 | 0.27 | 11.3 | This study |

Notes: [a] Only the results of batch fermentation were adopted.

(Fig. 5e). The lipid production efficiency reached over 82.0% of the theoretical yield and 11.3 g/L/d of productivity by *T. cutaneum* MS28. The performance of lipid production from lignocellulose feedstock by *T. cutaneum* MS28 was compared to the previous studies of cellulosic lipid production (Table 2). *T. cutaneum* MS28 exhibits better performances in lipid titer, yield, and productivity. Although some studies obtained higher lipid titer by fed-batch fermentation [42,49], the preparation of lignocellulosic hydrolysate containing high concentrations of sugars generally involved energy-consuming solid/liquid separation and concentration operations, leading to the increase in costs.

Since the evolved strain *T. cutaneum* MS28 showed significantly enhanced sugars metabolism and lipid accumulation compared to the parental strain, the changes in the expression levels of the key genes related to lignocellulose-derived sugars metabolism and lipid synthesis were investigated (Fig. 6). Overall, the expression levels of the genes associated with the metabolism of sugars were up-regulated, which is different from the results of other studies that the presence of phenolics lowered the genes expression levels of glucose uptake and consumption in *Clostridium acetobutylicum* [28,29].

Significant changes in the expression levels of the key genes in lipid synthesis were also observed. The expression levels of the selected genes responsible for fatty acid and triacylglycerol were up-regulated by 3–89 folds. The supply of lipid synthesis precursors was also enhanced. The expression level of gene *tpiA* encoding triosephosphate isomerase was up-regulated by 130.9-fold, which facilitated the efficient synthesis of

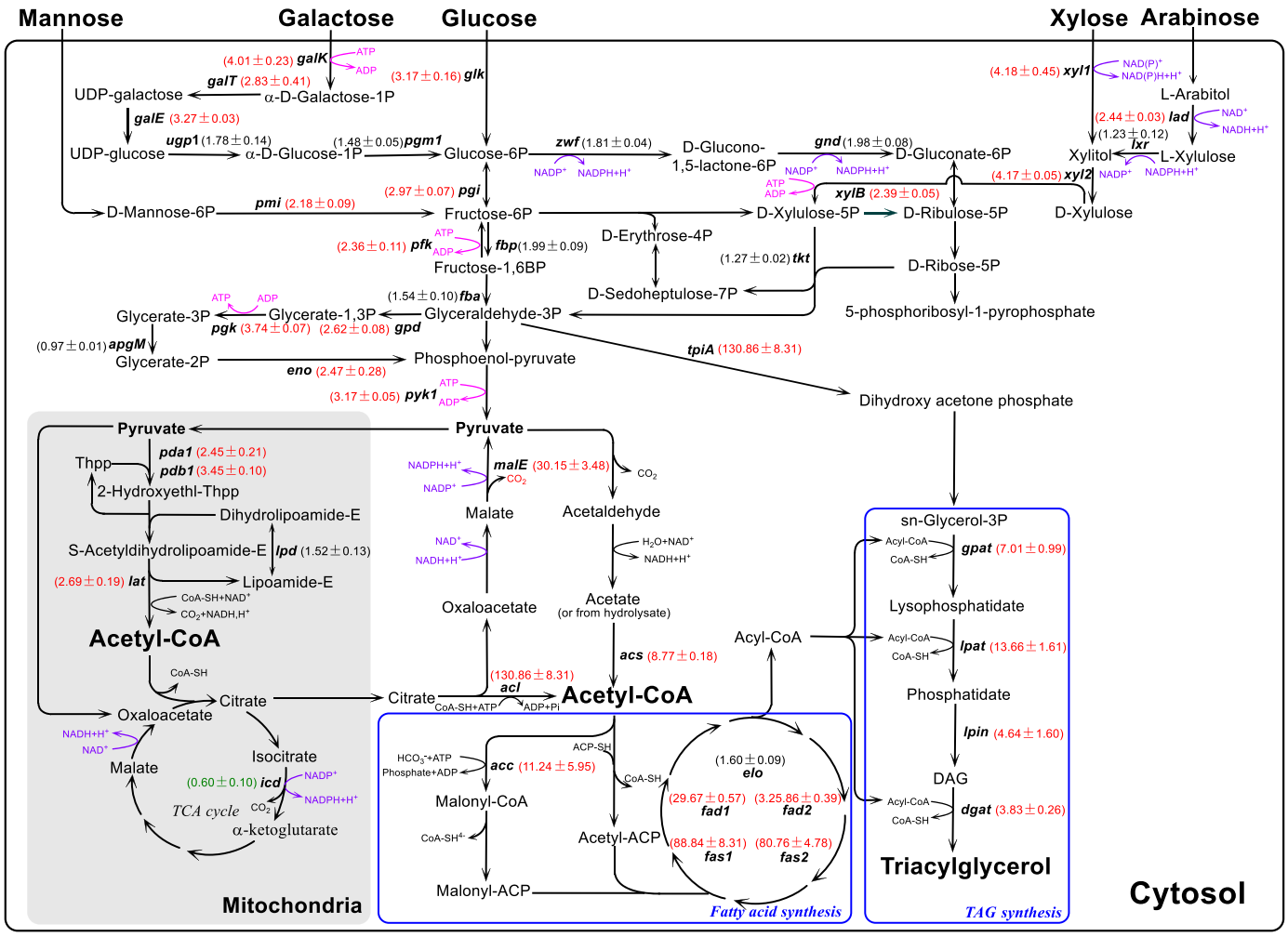


**Fig. 6.** Transcriptional analysis of the genes involved in central carbon metabolism of lipid biosynthesis in *T. cutaneum* MS28 compared to the parental strain. The samples were collected after cultured in 20% (w/w) solids loading corn stover clarified hydrolysate for 24 h. The gene expression levels were normalized against the parental strain *T. cutaneum* ACCC 20271. Each RT-qPCR was performed at least three times.

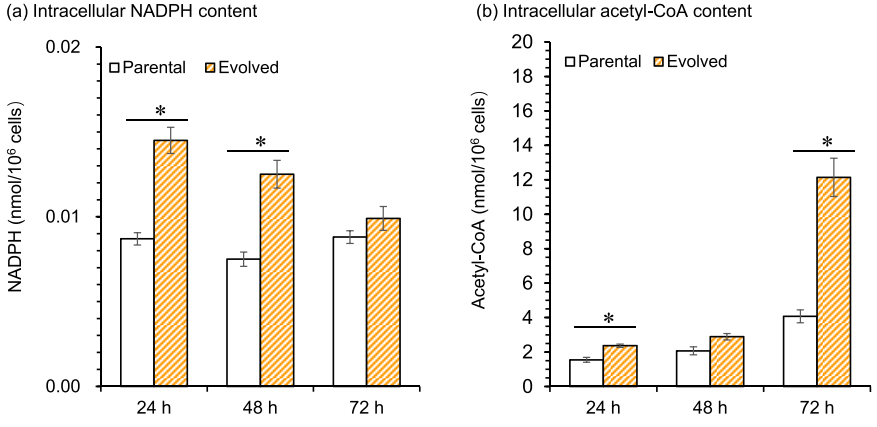


**Fig. 7.** Contents of key precursors for lipid synthesis in *T. cutaneum* cells. (a) NADPH; (b) acetyl-CoA. The samples for measurement were cultured in the 20% (w/w) solids loading corn stover hydrolysate and harvested at 24 h, 48 h, and 72 h intervals.

the lipid precursor dihydroxy acetone phosphate from glyceraldehyde-3-phosphate. The significantly up-regulated (8–131 folds) genes *acl*, *acs*, and *malE* encoding ATP-dependent citrate lyase gene, acetyl-CoA synthetase gene, and malic enzyme contributed to the generation of more acetyl-CoA and NADPH as lipid precursors [10]. The contents of intracellular metabolites including acetyl-CoA and NADPH were further measured to confirm the transcriptional analysis results (Fig. 7). The content of NADPH in *T. cutaneum* MS28 was approximately 1.7-fold greater than that in parental strain at 24 h (Fig. 7a). And the intracellular content of acetyl-CoA in *T. cutaneum* MS28 was approximately 3.0-fold greater than that in parental strain at 72 h (Fig. 7b).

In summary, after the long-term adaptive evolution combined with

centrifugal enrichment in partially biodetoxified corn stover hydrolysate, the finally obtained strain *T. cutaneum* MS28 showed bigger cell volume, higher lipid accumulation, enhanced phenolic aldehyde inhibitors tolerance, increased lipid synthesis precursors generation, more active fermentable sugars metabolism and lipid synthesis shunts, which collectively provided the positive factors for cellulosic lipid production. On the other hand, this study also provides numerous gene targets for improving the phenolic aldehyde inhibitors and lipid accumulation in oleaginous yeast. The function of these genes discovered in this study will be further verified by heterologous expression in the future work.

## 4. Conclusions

The adaptive evolution combined with centrifugal enrichment in lignocellulosic hydrolysate was applied to simultaneously improve the lignin-derived inhibitors tolerance and the lipid accumulation in oleaginous yeast *T. cutaneum*. The evolved strain *T. cutaneum* MS28 showed larger cell volume, higher cell growth and lipid content, enhanced phenolic aldehyde inhibitors tolerance, and improved cellulosic lipid production. Transcriptional analysis further showed that the evolved strain had more active fermentable sugars metabolism and lipid synthesis shunts. This adaptive evolution in lignocellulose hydrolysate combined with centrifugal enrichment is an efficient tool to targeted enhancement of inhibitors tolerance and cellulosic lipid production of oleaginous yeast.

## CRediT authorship contribution statement

**Jie Bao:** Writing – review & editing, Writing – original draft, Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Mingshan Hu:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bin Zhang:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Qi Liu:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Acknowledgments

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