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# Tolerance of *Trichosporon cutaneum* to lignin derived phenolic aldehydes facilitate the cell growth and cellulosic lipid accumulation

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ABSTRACT

Phenolic aldehydes are the major inhibitors from lignocellulose pretreatment. Previous studies show that oleaginous yeasts are difficult to survive in lignocellulosic hydrolysates even after the removal of furan aldehydes and organic acids inhibitors. This study investigated the cell viability, sugar consumption and lipid accumulation of the major oleaginous yeasts including *Trichosporon cutaneum*, *Rhodosporidium toruloides*, *Rhodotorula glutinis*, *Yarrowia lipolytica* in wheat straw hydrolysate containing only phenolic aldehydes after furan aldehydes and organic acids were selectively degraded by microorganisms. The results confirmed that the existence of residual phenolic aldehydes was the major reason for poor cell growth and metabolism of oleaginous yeasts. Only *T. cutaneum* demonstrated the higher tolerance by biodegrading phenolic aldehydes and the satisfactory cell growth and lipid production were obtained. This study revealed that *T. cutaneum* might be one of the promising cell factories for microbial lipid production from lignocellulosic feedstock.

## 1. Introduction

Microbial lipid production from lignocellulose by oleaginous yeasts is one of the most promising technologies for sustainable supply for aviation fuel and biodiesel feedstocks (Huang et al., 2013; Nogue et al., 2018; Poontawee et al., 2017; Patel et al., 2016). However, one crucial problem in microbial lipid fermentation using lignocellulose biomass as feedstock is that oleaginous yeasts are generally hard to survive in lignocellulosic hydrolysate, resulting the microbial lipid production far below the required performance for practical applications (Jin et al., 2015; Osorio-Gonzalez et al., 2019; Perna et al., 2018; Yu et al., 2020). Inhibitors are generated from pretreatment of lignocellulose, and their variety and contents depend on pretreatment method and material used (Jonsson et al., 2013; Saini et al., 2020; Valdes et al., 2020; Yu et al., 2011). In order to improve the survival environment of fermentation strains, the detoxification step is followed to remove the inhibitors (Kim, 2018), such as over-liming (Martinez et al., 2001), adsorption (Travalia et al., 2019), ion exchange (Llano et al., 2017), biodetoxification (Matsakas et al., 2017; Tramontina et al., 2020; Zhang et al., 2010b). While the furan aldehydes inhibitors (furfural and 5-hydroxymethylfurfural) and organic acids (acetic acid, levulinic acid, formic acid) are capable of complete removal by biological detoxification (He et al., 2016; Zhang et al., 2010b), phenolic aldehyde are difficult to be removed completely (Gu et al., 2015; Kondaveeti et al., 2019; Thomsen et al., 2009). Therefore, a certain number of phenolic aldehydes residues still exist in pretreated lignocellulose even after detoxification step. It is suspected that the existence of residual phenolic aldehydes is responsible for the poor viability of oleaginous yeasts, but not yet verified.This study experimentally investigated the existence of phenolic aldehydes on the viability and metabolism of several oleaginous yeasts used as the major fermentation strains for cellulosic lipid production. The speculation of phenolic aldehydes inhibition on oleaginous yeasts was confirmed. *T. cutaneum* was found as highly tolerant to phenolic aldehydes by efficiently degradation of phenolics. The cellulosic lipid production by *T. cutaneum* was confirmed to be the most adaptive cell factory for microbial lipid production from lignocellulosic feedstock.

## 2. Materials and methods

### 2.1. Strains, media and culture conditions

*Yarrowia lipolytica* DSM 3286 was obtained from German National Resource Centre for Biological Material, Braunschweig, Germany. *Rhodotorula glutinis* CGMCC 2.703, *Rhodosporidium toruloides* CGMCC 2.1609 was obtained from China General Microorganism Collection Center (CGMCC), Beijing, China. *Trichosporon cutaneum* ACCC 20271

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was obtained from Agricultural Culture Collection of China (ACCC), Beijing, China. *Trichosporon cutaneum* MP11 was the mutant strain of *T. cutaneum* ACCC 20271 and registered in CGMCC with the number 20481. Oleaginous yeasts were activated at 30 ℃ in YPD medium, and cultured in the synthetic medium (SM) containing 1.0 g/L yeast extract, 1.0 g/L $KH_2PO_4$, 0.5 g/L $MgSO_4.7H_2O$, 0.22 g/L $(NH_4)_2SO_4$.*Paecilomyces variotii* FN89 (registration number 17665) was used for biodetoxification and stored in CGMCC. The culture condition was 37 ℃ in the seed medium containing 20 g/L glucose, 2 g/L $KH_2PO_4$, 1.0 g/L $MgSO_4.7H_2O$, 1.0 g/L yeast extract.

### 2.2. Enzymes and reagents

Cellulase was purchased from Novozymes China (Beijing, China) with the filter paper, cellobiase activity and protein concentration activity, of 203.2 FPU/mL, 4900 CBU/mL and 87.3 mg/mL (Adney and Baker, 1996; Bradford, 1976; Ghose, 1987). Glucose and other chemicals were purchased from Titan, Shanghai, China.

### 2.3. Wheat straw and its biorefinery operations

The 35.2% (w/w) of cellulose and 22.4% (w/w) of hemicellulose in raw wheat straw were determined according to the protocols of NREL (Sluiter et al., 2008a, 2008b). The raw wheat straw was dry acid pretreated with 3.5% (w/w) $H_2SO_4$ (3.5 g per gram of100 g dry solid matter) used as catalyst (He et al., 2014; Zhang et al., 2011). Briefly, the wheat straw and sulfuric acid solution were uniformly mixed in pretreatment reactor at solid-to-liquid ratio of 2:1 (w/w), and then the hot steam was fed into pretreatment reactor and maintained at 175 ℃ for 5 min. Biodetoxification was carried out by inoculating *P. variotii* FN89 on the pretreated wheat straw (Chinese Invention Patent, license number ZL201911039157.4). Wheat straw hydrolysate at 15% (w/w) solids contents was prepared with 5.5 mg cellulase proteins/g cellulose used for hydrolysis (Wang et al., 2016). Briefly, the pretreated and biodetoxified wheat straw was enzymatically hydrolyzed at pH 4.8, 50 °C for 48 h. The wheat straw hydrolysate was centrifuged at 10,000 rpm for 5 min to collect the clear supernatant, and then the filtered wheat straw hydrolysate was added with 1.0 g/L $KH_2PO_4$, 1.0 g/L yeast extract, 0.5 g/L $MgSO_4.7H_2O$, and 0.22 g/L $(NH_4)_2SO_4$.

### 2.4. Lipid fermentation

Lipid fermentation was conducted at 30 ℃ and pH 5.0 with 10% (v/v) inoculate ratio. Simultaneous saccharification and lipid co-fermentation (SSCF) was performed as described in Liu et al. (2012) and Zhang et al. (2010a). The pretreated and biodetoxified wheat straw at 30% (w/w) solids contents was pre-hydrolyzed at 50 ℃, pH 4.8 for 12 h by adding 5.5 mg cellulase proteins/g dry wheat straw, and then *T. cutaneum* MP11 seeds at 10% (v/v) inoculation was added to initiate the SSCF at 30 ℃, pH 5.0.

### 2.5. Analytical methods

The dry cell weight (DCW) was measured as described in Hu et al. (2018). The lipid was extracted as described in Folch et al. (1957) and Hu et al. (2018). The total phenolic content was measured by a modified Folin & Ciocalteu method described in Ainsworth and Gillespie (2007) and Gu et al. (2014).

Glucose, xylose, organic acid, furan aldehydes and phenolic compounds were analyzed using an HPLC (Yi et al., 2019).

## 3. Results and discussion

### 3.1. Phenolic aldehydes inhibited the cell growth and metabolism of oleaginous yeasts

To evaluate the cell growth and lipid accumulation of oleaginous yeasts using lignocellulose as feedstock, T. *cutaneum* ACCC 20271, *T. cutaneum* MP11, *Y. lipolytica* DSM 3286, *R. glutinis* CGMCC 2.703, and *R. toruloides* CGMCC 2.1609 were cultured in the wheat straw hydrolysate, in which the biodetoxification removed furfural, 5-hydroxymethylfurfural (HMF), acetic acid till not detected level using HPLC. Phenolic inhibitors are derived from the degradation of lignin and the representatives include 4-hydroxybenaldehyde, 4-hydroxy-3-methoxybenzaldehyde and syringaldehyde, and they are the primary objects of investigation in considering the inhibition from phenolic inhibitors. After biodetoxification, the phenolic aldehydes were still in considerably high levels (Fig. 1), with the representative phenolic compounds 4-hydroxybenzaldehyde (HBA), 4-hydroxy-3-methoxybenzaldehyde, syringaldehyde of 60.21 mg/L, 79.62 mg/L, 30.34 mg/L, respectively, and the total phenolics content of 690 mg/L. Actually, the complete removal of phenolic inhibitors was almost not possible by physical and chemical detoxification methods, and the biodetoxification removal led to the large loss of xylose (Gu et al., 2014; Palmqvist and Hahn-Hägerdal, 2000; Liu et al., 2020).

Fig. 2 shows the lipid accumulation of the five major oleaginous yeasts using wheat straw hydrolysate as carbon source, in which only residual phenolics inhibitors were left behind after biodetoxification. *Y. lipolytica* DSM 3286, *R. toruloides* CGMCC 2.1609, and *R. glutinis* CGMCC 2.703 were poor in all the measurable parameters, while the two *T. cutaneum* strains showed the obvious advantages on cell growth and lipid accumulation. These results reveal that general oleaginous yeasts, except *T. cutaneum*, might be sensitive to phenolic aldehydes.

The inhibition of phenolic aldehydes on the cell growth of oleaginous yeasts was further examined by adding HBA, 4-hydroxy-3-methoxybenzaldehyde, or syringaldehyde into the synthetic medium (Fig. 3). Again, only the two *T. cutaneum* strains (ACCC 20271 and MP11) showed the satisfactory cell growth, but almost no cell growth was observed for *Y. lipolytica* DSM 3286, *R. toruloides* CGMCC 2.1609, and *R. glutinis* CGMCC 2.703. These results confirmed that the phenolic aldehydes significantly inhibited the cell viability and metabolism of various oleaginous yeasts, while *T. cutaneum* showed the best tolerance to phenolic aldehydes.

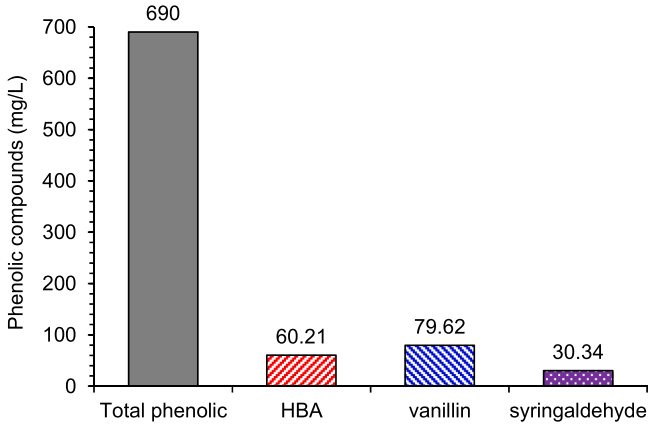


**Fig. 1.** Contents of the total phenolics and the representative phenolics (HBA, 4-hydroxy-3-methoxybenzaldehyde and syringaldehyde) in the biodetoxified wheat straw hydrolysate.

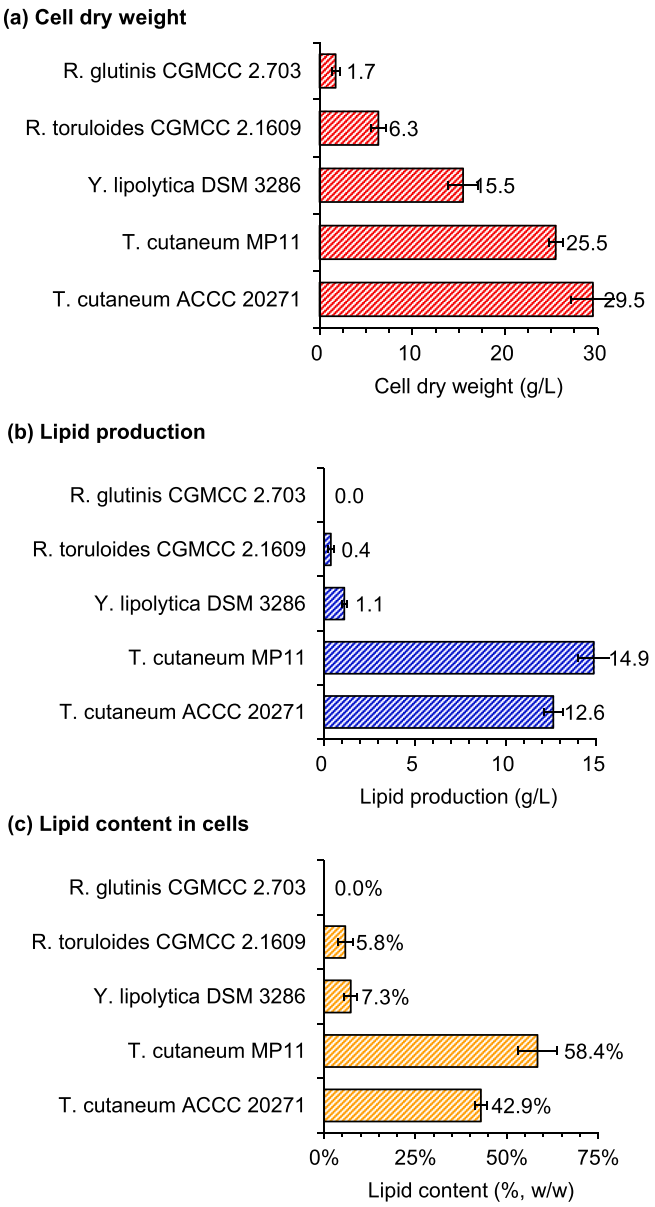


**Fig. 2.** Lipid fermentation of five oleaginous yeasts in wheat straw hydrolysate after biodetoxification. (a) Dry cell weigh; (b) Lipid production; (c) Lipid contents in cells. 50 mL wheat straw hydrolysate in 500 mL flasks, 180 rpm, 120 h.

### *3.2. Bioconversion of phenolic aldehydes to less toxic derivatives by oleaginous yeasts*

Microbial tolerance of phenolic aldehydes is generally understood by its capacity of phenolic aldehydes degradation (Liu et al., 2009). Therefore, the bioconversion of HBA, 4-hydroxy-3-methoxybenzaldehyde, and syringaldehyde by oleaginous yeasts were examined. Fig. 4 shows that the two *T. cutaneum* strains (ACCC 20271 and MP11) almost completely consumed HBA, 4-hydroxy-3-methoxybenzaldehyde, or syringaldehyde into their alcohol and acid derivatives, then the alcohols or acids were also efficiently degraded (Fig. 4**a** and **b**). On the other hand, *Y. lipolytica* DSM 3286, *R. glutinis* CGMCC 2.703, and *R. toruloides* CGMCC 2.1609 only degraded a small portion of HBA, 4-hydroxy-3-methoxybenzaldehyde, or syringaldehyde into the corresponding acids or

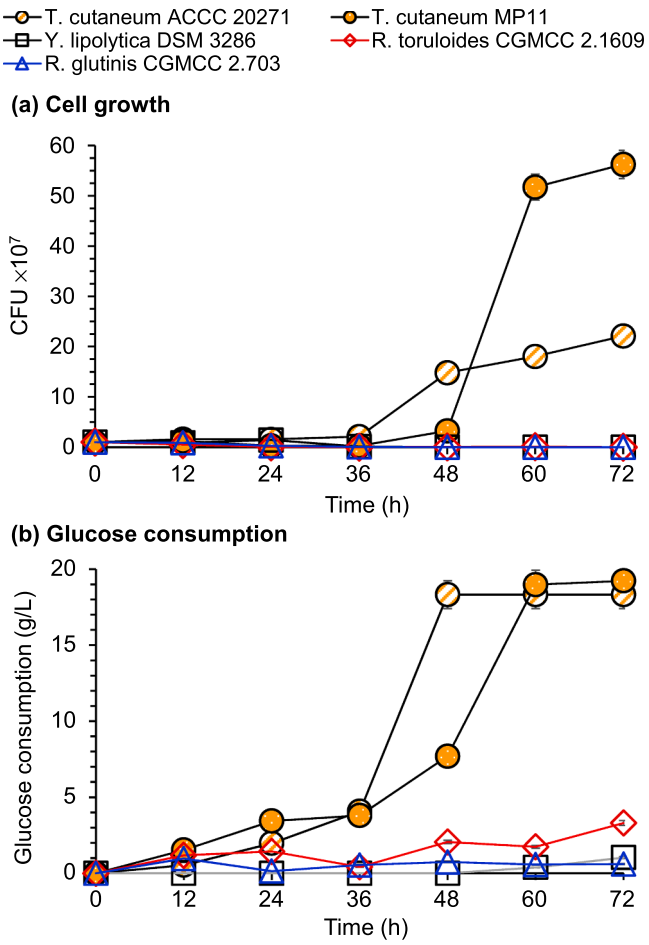


**Fig. 3.** Cell growth and glucose consumption of oleaginous yeasts under the stress of phenolic aldehydes. 800 mL synthetic medium (20 g/L glucose, 0.8 g/L HBA, 0.8 g/L 4-hydroxy-3-methoxybenzaldehyde and 0.6 g/L syringaldehyde) in 3 L bioreactor, 10% (v/v) inoculate ratio, 1 vvm, 450 rpm.

alcohols, and then ceased the further conversion (Fig. 4**c, d, and e** ). The result revealed that the high phenolic aldehydes tolerance of *T. cutaneum* came from the strong capacity of phenolic aldehydes conversion, while the weak tolerance of *Y. lipolytica*, *R. glutinis*, and *R. toruloides* were due to their weak bioconversion capacity of phenolic aldehydes. The putative bioconversion pathway (Fig. S1) of three representative phenolic by *T. cutaneum* was constructed based on the previous results (Hu et al., 2018; Kosa and Ragauskas, 2013; Wang et al., 2016). *T. cutaneum* first converts HBA, 4-hydroxy-3-methoxybenzaldehyde and syringaldehyde to its alcohols form in a reversible reaction by enzymatic catalysis, which leaves aldehydes at a lower level, then further oxidizes the aldehydes to corresponding acid through irreversible reaction. Finally, 4-hydroxybenzyl acid is converted into acetyl CoA and succinyl CoA used as the precursors for lipid synthesis, whereas vanilic acid and syringic acid might not be able to flux into the lipid synthesis pathway based on the experimental results of no lipid accumulation when 4-hydroxy-3-methoxybenzaldehyde or syringaldehyde is used for cultivation of *T. cutaneum* (data not shown). The possible end-products of 4-hydroxy-3-methoxybenzaldehyde and syringaldehyde degradation might be some melanin precursors through the formation of catechol as intermediate.

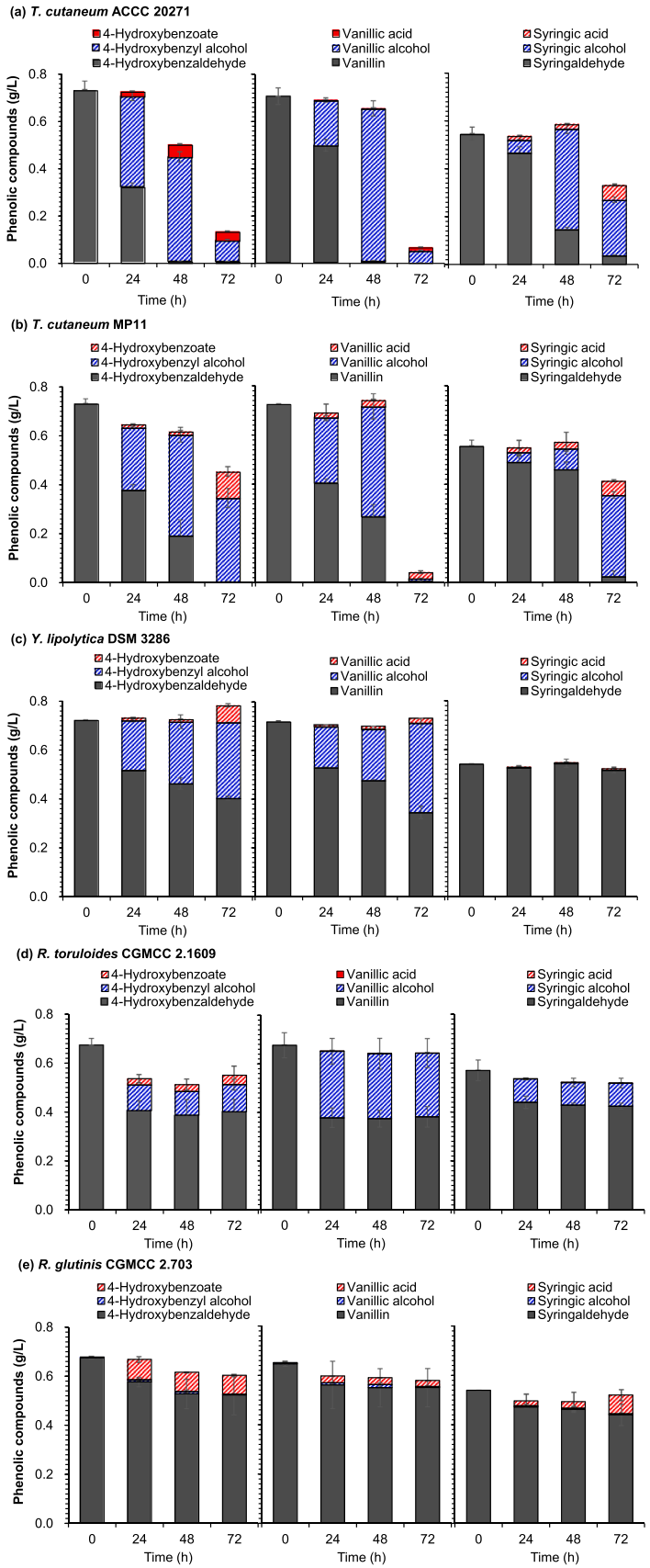


**Fig. 4.** Biodegradation of phenolic aldehyde by oleaginous yeasts. (a) *T. cutaneum* 20271; (b) *T. cutaneum* MP11; (c) *Y.lipolytica* DSM 3286; (d) *R. toruloides* CGMCC 2.1609; (e) *R. glutinis* CGMCC 2.703. 800 mL synthetic medium containing 20 g/L glucose and phenolic aldehyde (0.8 g/L HBA, 0.8 g/L 4-hydroxy-3-methoxybenzaldehyde and 0.6 g/L syringaldehyde) in 3 L bioreactor, 10% (v/v) inoculate ratio, 450 rpm, 1 vvm, pH 5.0.

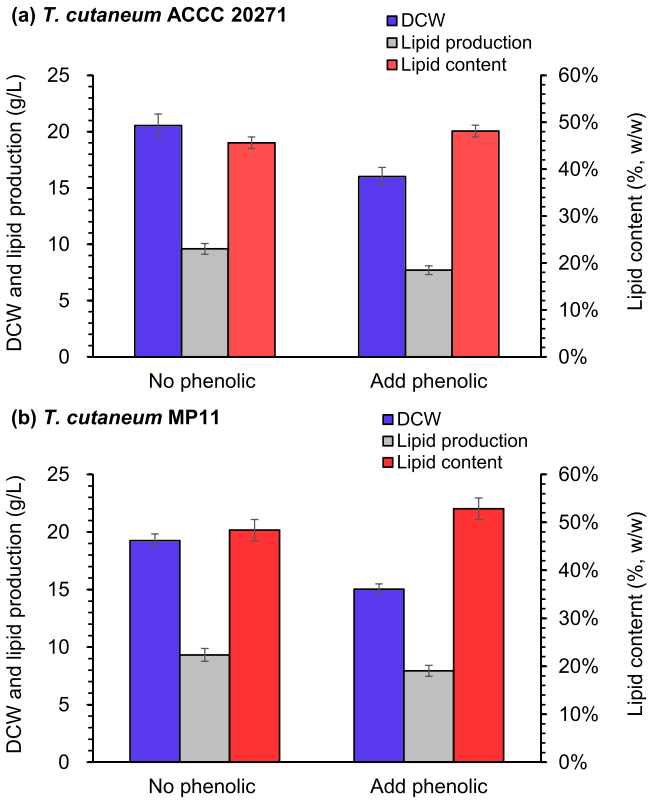


**Fig. 5.** Lipid fermentation evaluation of *T. cutaneum* in the presence of phenolic aldehydes. (a) *T. cutaneum* 20271; (b) *T. cutaneum* MP11. 50 mL synthetic medium containing 60 g/L glucose, 0.8 g/L HBA, 0.8 g/L 4-hydroxy-3-methoxybenzaldehyde and 0.6 g/L syringaldehyde in 500 mL flasks, 30 °C, 180 rpm, 120 h.

### *3.3. Cellulosic lipid production of T. cutaneum under tolerance of phenolic aldehydes*

*T. cutaneum* showed high phenolic aldehydes tolerance by its strong capacity phenolic aldehydes bioconversion. Then the lipid accumulation performance of *T. cutaneum* in the presence of phenolic aldehydes was investigated (Fig. 5). The results show that although the cell mass of *T. cutaneum* 20271 and MP11 were reduced by approximately 20% at the typical levels of phenolic aldehydes presence, the lipid content in yeast cells were almost not affected by the existence of phenolics. The final lipid production decreased by only 19.7% for ACCC 20271 and 14.9% for MP11, respectively. No phenolic aldehydes were detected after lipid fermentation. Clearly, the lipid production by *T. cutaneum* was less affected by the existence of phenolic aldehydes at the typical phenolics content levels. In addition, *T. cutaneum* showed better lipid fermentation performance than *T. cutaneum* ACCC 20271. Then *T. cutaneum* MP11 was applied to lipid fermentation using wheat straw as feedstock by SSCF (Fig. 6). The initial total phenolic content in the whole wheat straw hydrolysate was determined to be 9.49 g/L. The record high lipid production of 40.87 ± 1.85 g/L was obtained with complete conversion of both glucose and xylose after 144 h. The tolerance of oleaginous yeast to inhibitors is essential for cellulosic lipid production. Chen et al. (2009) showed that *T. cutaneum* was a well adaptive strain to various inhibitors from lignocellulose. Gao et al. (2014) confirmed that only *T. cutaneum* accumulated lipid in the corncob residues hydrolysate. This study reveals that the residual phenolic aldehydes in the biodetoxified lignocellulose feedstock, even after efficient biodetoxification, still inhibit the cell growth and lipid metabolism of many oleaginous yeasts. Again, *T. cutaneum* showed the unique exception of the phenolics inhibitions as the promising oleaginous yeast for lipid production using lignocellulosic feedstock.

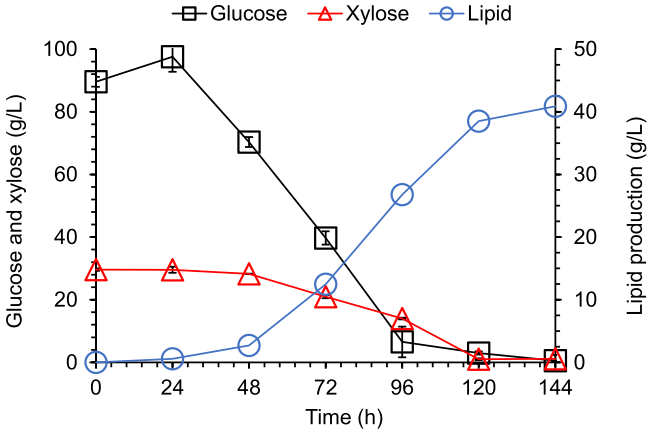


**Fig. 6.** Simultaneous saccharification and co-fermentation (SSCF) of *T. cutaneum* MP11 for lipid production. 30 °C, 450 rpm, 1 vvm, pH was controlled at 5.0.

## 4. Conclusion

Phenolic aldehydes were verified to be the reason for poor cell growth and lipid accumulation of oleaginous yeast in the detoxified lignocellulosic feedstock. *T. cutaneum* showed high tolerance to phenolic aldehydes and was able to degrade the three typical phenolic aldehydes (4-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde, syringaldehyde) into their acids form and then further degraded acids. The lipid metabolism of *T. cutaneum* was almost not affected by the existence of phenolic aldehydes and the 40.87 ± 1.85 g/L of lipid was produced by SSCF.

## CRediT authorship contribution statement

Yi Zhang: Designing and preforming the experiment; Writing the manuscript. Jie Bao: Designing and monitoring the experiment, Writing - review & editing the manuscript.

## 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.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2021.09.009.

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