



# Inhibitor degradation and lipid accumulation potentials of oleaginous yeast *Trichosporon cutaneum* using lignocellulose feedstock



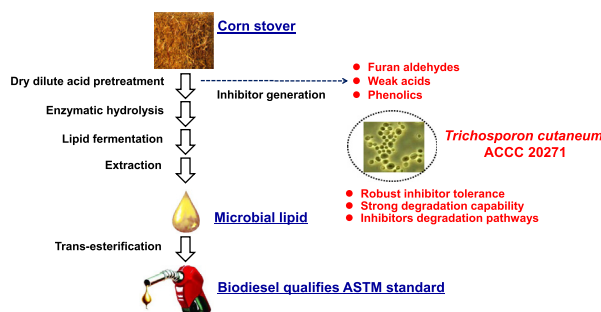
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## HIGHLIGHTS

- *T. cutaneum* accumulates high lipid using lignocellulose feedstock.
- Synthesized biodiesel using the lipid meets the ASTM standard.
- *T. cutaneum* is tolerant to high levels of inhibitors.
- *T. cutaneum* quickly degrades all major inhibitors.
- Inhibitor degradation pathways of *T. cutaneum* are constructed.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Oleaginous yeast *Trichosporon cutaneum* is robust to high levels of lignocellulose derived inhibitor compounds with considerable lipid accumulation capacity. The potential of lipid accumulation of *T. cutaneum* ACCC 20271 was investigated using corn stover hydrolysates with varying sugar and inhibitor concentrations. Biodiesel was synthesized using the extracted lipid and the product satisfied the ASTM standards. Among the typical inhibitors, *T. cutaneum* ACCC 20271 is relatively sensitive to furfural and 4-hydroxybenzaldehyde, but strongly tolerant to high titers of formic acid, acetic acid, levulinic acid, HMF, vanillin, and syringaldehyde. It is capable of complete degradation of formic acid, acetic acid, vanillin and 4-hydroxybenzaldehyde. Finally, the inhibitor degradation pathways of *T. cutaneum* ACCC 20271 were constructed based on the newly sequenced whole genome information and the experimental results. The study provided the first insight to the inhibitor degradation of *T. cutaneum* and demonstrated the potentials of lipid production from lignocellulose.

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## 1. Introduction

Oleaginous yeasts such as *Trichosporon cutaneum*, *Lipomyces starkeyi*, *Rhodospiridium toruloides*, *Rhodotorula glutinis*, *Trichosporon fermentans*, *Yarrowia lipolytica* accumulate over 20% intracellular lipid of their cell mass (Sitepu et al., 2014). The lipid produced is considered as an important alternative of vegetable oil feedstock for biodiesel production, if the regular starch or sucrose feedstock is replaced by a cheaper feedstock. Among many

options, lignocellulose biomass shows great potential for its great abundance and biorefining technology advances. Various oleaginous yeast strains have exhibited the potential of growth and microbial lipid production from lignocellulose feedstock, although it is still at the initial stage of practical applications (Papanikolaou and Aggelis, 2011; Huang et al., 2013). In biorefinery processing of lignocellulose, a harsh pretreatment step is required to disrupt its rigorous structure of lignocellulose biomass before enzymatic hydrolysis. The typical pretreatment methods include dilute acid, steam explosion, ammonia fiber explosion, alkaline, ionic liquid etc. (Mosier et al., 2005). Each method inevitably generates various inhibitor compounds to cellulose enzyme and fermenting

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microorganisms, including furans (furfural, 5-hydroxymethylfurfural (HMF)), phenolic compounds (vanillin, syringaldehyde, 4-hydroxybenzaldehyde) and weak acids (formic acid, acetic acid, levulinic acid) (Palmqvist and Hahn-Hagerdal, 2000; Klinke et al., 2004; Almeida et al., 2007). The inhibitor tolerance is considered as the most immediate technical barrier for lipid production from lignocellulose (Jin et al., 2015). Therefore, oleaginous yeasts using lignocellulose derived sugar feedstocks should not only accumulate high intracellular lipid, but also be tolerant to various inhibitors.

*T. cutaneum* is an oleaginous yeast capable of utilizing various sugars such as glucose, xylose, cellobiose, lactose (Hu et al., 2011; Mörtberg and Neujahr, 1986) and tolerant to high levels of lignocellulose derived inhibitors (Chen et al., 2009). Lipid fermentation by *T. cutaneum* using lignocellulose feedstocks such as corn stover hydrolysate (Liu et al., 2012), corncob residues hydrolysate (Gao et al., 2014), corncob acid hydrolysate (Chen et al., 2013) had been investigated. Recently, we sequenced the whole genome of *T. cutaneum* ACCC 20271 and deposited at DDBJ/EMBL/GenBank with the accession number LTAL000000000 (<http://www.ncbi.nlm.nih.gov/nucleotide/acc20271>) (Wang et al., 2016). The genome information gives the molecular basis for prediction of microbial oleaginicity and inhibitor tolerance.

In this study, the maximum lipid accumulation potentials of *T. cutaneum* ACCC 20271 using lignocellulose feedstock and its inhibitor tolerance and degradation pathways to eight typical inhibitors were investigated based on the experimental data, the genome annotation, and the previous relevant studies. This study provides the first insight into the biodegradation of lignocellulose derived inhibitors by oleaginous yeast for lipid fermentation potential as the biodiesel feedstock for future industrial applications.

## 2. Materials and methods

### 2.1. Raw materials

Corn stover was harvested in fall 2012 from Nanyang, Henan, China. The corn stover was milled and passed through the round screens with 10 mm in diameter, then washed to remove field dirt, stones and metals, and dried to a constant weight. The raw corn stover contained 38.72% of cellulose, 20.55% of hemicellulose, 26.51% of lignin, 2.76% of ash on dry weight base (w/w) determined by two-step sulfuric acid hydrolysis according to NREL Laboratory Analytical Procedure (LAP) protocols (Sluiter et al., 2008a,b).

### 2.2. Enzymes and reagents

The cellulase enzyme Youtell #6 was purchased from Hunan Youtell Biochemical Co., Yueyang, Hunan, China. The filter paper activity was 63.0 FPU/g according to the NREL LAP-006 (Adney and Baker, 1996), and the cellobiase activity was 102.0 CBU/g according to the method of Ghose (1987).

Yeast extract was purchased from Oxoid, Basingstoke, Hampshire, UK. 2-Furaldehyde (furfural), 5-hydroxymethylfurfural (HMF) and furan-2,5-dicarboxylate were from J&K Scientific, Beijing, China. Furfuryl alcohol, 2-furoic acid, 5-hydroxymethylfurfural-2-furoate (HMF acid) and 5-hydroxymethylfurfuryl alcohol (HMF alcohol) were from Bide Pharmatech, Shanghai, China. Vanillin and 4-hydroxybenzaldehyde (HBA) were from Aladdin Reagents and Sangon Biotech, Shanghai, China. Vanillyl alcohol, vanillate, 4-hydroxybenzyl alcohol and 4-hydroxybenzoate were from Tokyo Chemical Industry, Tokyo, Japan. Syringaldehyde, syringyl alcohol and syringate were from Alfa Aesar, Heysham, UK. Formic acid, acetic acid, and levulinic acid were from Sinopharm

Chemical Reagent, Shanghai, China. All other chemicals including glucose, peptone,  $\text{KH}_2\text{PO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{MgSO}_4$ ,  $\text{NaOH}$ ,  $\text{H}_2\text{SO}_4$ , boron trifluoride, were of analytical reagent grade and purchased from Lingfeng Chemical Reagent Co., Shanghai, China.

### 2.3. Strains and media

*T. cutaneum* ACCC 20271 was stored in Agricultural Culture Collection of China (ACCC), Beijing, China (<http://www.accc.org.cn/>). The cells were cultured in YPD medium (20 g/L of glucose, 20 g/L of peptone and 10 g/L of yeast extract) at 30 °C for 12 h, then 10% of the culture broth was inoculated into the fresh YPD medium and cultured at 30 °C for 24 h as the seed culture of lipid fermentation in corn stover hydrolysates.

*Amorphotheca resinae* ZN1 was isolated in our previous work (Zhang et al., 2010) and stored in China General Microbiological Culture Collection Center (CGMCC, <http://www.cgmcc.net/>), Beijing, China under the accession number CGMCC 7452. *A. resinae* ZN1 was cultured in potato-dextrose agar (PDA) medium (200 g/L of potato extract juice, 20 g/L of glucose with 15 g/L of agar) at 28 °C.

Synthetic medium for inhibitors tolerance and degradation contained 0.5 g/L of yeast extract, 0.22 g/L of  $(\text{NH}_4)_2\text{SO}_4$ , 0.5 g/L of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 1.0 g/L of  $\text{KH}_2\text{PO}_4$ . The concentration of every inhibitor was chose according to pretreated corn stover with different pretreatment intensity (Zhang et al., 2011).

### 2.4. Pretreatment, biodegradation and saccharification

Corn stover was co-currently fed into a 20 L pretreatment reactor under helical agitation with 5% (w/w) diluted sulfuric acid solution at solid-to-liquid ratio of 2:1 (w/w) to reach the  $\text{H}_2\text{SO}_4$  usage of 2.5 g per gram of 100 g dry solid matter (DM) (Zhang et al., 2010; He et al., 2014). The hot steam was jetted into the reactor to initiate the pretreatment operation and maintained for 5 min at 175 °C. The pretreated corn stover contained approximately 50% (w/w) of water without free wastewater generation. The pretreated corn stover contained 39.47% of cellulose, 6.80% of hemicellulose, 6.57% of ash determined according to NREL LAP protocols (Sluiter et al., 2008a,b), as well as 5.13 mg of furfural, 3.38 mg of HMF, 1.97 mg of formic acid, 16.65 mg of acetic acid, 2.54 mg of levulinic acid, 1.27 mg of vanillin, 0.67 mg of syringaldehyde, and 0.18 mg of 4-hydroxybenzaldehyde per gram of dry solid matter.

The sulfuric acid in the pretreated corn stover was neutralized to pH 5.5–6.0 by adding 20% (w/v)  $\text{Ca}(\text{OH})_2$  slurry, then biodegraded at 28 °C for 5 days by inoculating *Amorphotheca resinae* ZN1 (Zhang et al., 2010; He et al., 2016). The inhibitors per gram of dry pretreated corn stover after biodegradation included 1.33 mg of furfural, 1.43 mg of HMF, 0.57 mg of formic acid, 8.58 mg of acetic acid, 2.23 mg of levulinic acid, 0.62 mg of vanillin, 0.48 mg of syringaldehyde, and 0.08 mg of 4-hydroxybenzaldehyde per gram of dry solid matter.

The pretreated corn stover was enzymatically hydrolyzed using 15 FPU/g DM of cellulase at 50 °C, pH 4.8 for 72 h, then the supernatant liquid was centrifuged, autoclaved and filtered before use. The sugar and inhibitor compositions of the hydrolysates prepared under different feedstocks (the freshly pretreated and the biodegraded) and solids contents (15%, 20%, and 25%, w/w) were shown in Table 1.

### 2.5. Lipid fermentation

Lipid fermentation was carried out in a 3 L bioreactor (Baoning Biotech, Shanghai, China) with working volume of 1 L at 30 °C, pH 5.0, aeration rate of 1.67 vvm, and the stirring rate at 600 rpm. The inoculation size of the seed broth was 10% (v/v) of the total fermentation liquid. 0.5 g/L of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0 g/L of  $\text{KH}_2\text{PO}_4$ ,

**Table 1**  
Corn stover hydrolysates used.

Feedstocks	Solids contents (w/w)	Glucose (g/L)	Xylose (g/L)	Furfural (g/L)	HMF (g/L)	Acetic acid (g/L)	Vanillin (g/L)	Syringaldehyde (g/L)	HBA (g/L)
Freshly pretreated corn stover	15%	54.83	25.31	0.22	0.27	4.36	0.20	0.16	0.01
	20%	80.44	34.03	0.53	0.51	4.58	0.24	0.17	0.01
	25%	88.76	43.83	0.65	0.55	5.83	0.19	0.13	0.01
Pretreated and biodetoxified corn stover	15%	62.22	11.20	0.06	0.09	1.45	0.00	0.04	0.00
	20%	81.84	19.11	0.18	0.25	3.00	0.00	0.05	0.00
	25%	104.02	24.61	0.20	0.40	3.93	0.03	0.04	0.00

Two types of the pretreated corn stover feed stocks were hydrolyzed at the solids contents of 15%, 20%, 25% (w/w): the freshly pretreated corn stover (containing relatively high level of inhibitors without detoxification), and the pretreated corn stover after biodetoxification. The cellulase enzyme dosage was 15 FPU/g DM (dry corn stover mass) and the hydrolysis lasted for 72 h at 50 °C, pH 4.8. HMF indicates 5-hydroxymethylfurfural, HBA indicates 4-hydroxybenzaldehyde.

0.22 g/L of  $(\text{NH}_4)_2\text{SO}_4$  and 0.5 g/L of yeast extract were supplemented into the hydrolysates.

Sampling of 30 mL of fermentation broth was centrifuged to harvest the yeast cells, then washed and dried at 80 °C to constant weight for the measurement of dry cell mass (DCW). The lipid in cells was extracted using chloroform-methanol method (Folch et al. 1957). Briefly, the dried cells were soaked in 4 M HCl and boiled for 10 min then cooled on ice. The slurry was mixed with 20 mL of methanol and chloroform solution (1:2, v/v), and vigorously shaken at 30 °C for 1 h. The lipid dissolving chloroform phase was obtained by centrifugation and finally the lipid was obtained by vacuum evaporation at 80 °C.

The extracted lipid was directly transmethyalted according to Zhang et al. (2008) for fatty acid composition determination. Briefly, the transmethyalted lipid sample were analyzed using gas chromatograph-mass spectrometer (GC-MS) Clarus 500, PerkinElmer with HP-5 column of 30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ , Helium gas at 1 mL/min. The initial oven temperature was 80 °C and maintained for 3 min, then increased to 280 °C at the gradient of 16 °C per min, and hold at 280 °C for 2 min.

## 2.6. Preparation of biodiesel

The raw lipid obtained was dehydrated at 105 °C for 2 h, then 60 mL methanol solution containing 4.5% (w/v) KOH (in methanol) was added to 300 mL of dried lipid and agitated for 30 min with heating. Another 60 mL methanol containing 4.5% (w/v) of KOH was added at 60 °C then agitated for 3 h. The mixture was transferred to a separation funnel for separation of biodiesel from the crude glycerol and methanol after it was cooled to ambient temperature. The methanol in the upper liquid phase was evaporated at 65 °C on a rotary evaporator, and the crude glycerol was washed out by water for several times in a separation funnel. The upper residual liquid was biodiesel and dried at 105 °C for 2 h to remove the residual water.

## 2.7. Sugar and inhibitor analysis

Glucose, xylose, formic acid, acetic acid and levulinic acid in the hydrolysate were analyzed using HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Japan) equipped a Bio-rad Aminex HPX-87H column with the sulfuric acid as the eluent (5 mM, flow rate at 0.6 mL/min) at the column temperature 65 °C.

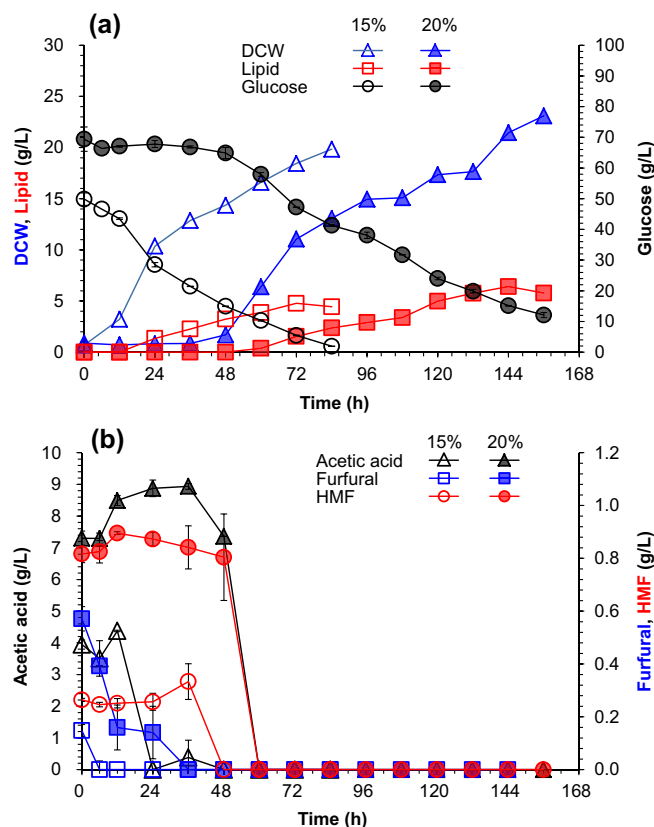
Furan and phenolic compounds were analyzed using HPLC (UV/Vis detector SPD-20A, Shimadzu, Kyoto, Japan) fitted with YMC-Pack ODS-A column (YMC, Kyoto, Japan) at 35 °C. The procedure for furfural, furfuryl alcohol and furoic acid the mobile phases was 50% (v/v) acetonitrile in water at flow rate of 1.0 mL/min and the detection wavelength of 220 nm. The gradient procedure applied for HMF, HMF alcohol and HMF acid: the mobile phases were solvent A (pure water) and solvent B (acetonitrile) at flow rate of 0.6 mL/min and the detection wavelength of 230 nm. Elu-

tion started 5% of eluent B and raised to 100% in 15 min, then it was decreased from 100 ~ 5% in 5 min and held at 5% for 10 min. The gradient procedure applied for phenolic compounds: the mobile phases were the eluent A (0.1% formic acid in water) and the eluent B (100% acetonitrile) at flow rate of 1.0 mL/min and the detection wavelength of 270 nm. Elution started at 10% of eluent B and raised to 35% in 4 min and held at 35% for 11 min, then, it was decreased from 35% to 10% in 5 min, and held at 10% of eluent B for 10 min.

## 3. Results and discussion

### 3.1. Lipid fermentation in corn stover hydrolysate and the consequent biodiesel synthesis

Maximum lipid accumulation of *T. cutaneum* ACCC 20271 using corn stover feedstock and its consequent biodiesel synthesis from



**Fig. 1.** Lipid fermentation of *T. cutaneum* ACCC 20271 using corn stover hydrolysates prepared from freshly pretreated corn stover. (a) Dry cell weight (DCW), lipid and glucose; (b) degradation of furfural, HMF and acetic acid. Culture conditions: pH 5.0, 30 °C, 600 rpm, aeration rate 1.67 vvm.

the extracted lipid were evaluated. Corn stover hydrolysates with different sugar concentrations prepared from two feedstocks (the freshly pretreated and the biodetoxified corn stover) and three solids contents (15%, 20% and 25%, w/w) were used as feedstock (Table 1). When the freshly pretreated corn stover was used as the hydrolysis feedstock, the hydrolysates prepared at 15% and 20% solids contents yielded 4.44 g/L and 5.78 g/L lipid (Fig. 1a), respectively, while furfural was completely degraded at 6 h and 36 h, HMF at 48 h and 60 h, acetic acid at 48 h and 60 h, respectively, in the two hydrolysates (Fig. 1b). The inhibitor level in the hydrolysate prepared at 25% (w/w) solids content seems exceed the maximum tolerance capacity and resulted in the extra-long lag time (over 96 h) with only minor cell growth and lipid accumulation. When the pretreated corn stover was biodetoxified to remove most of the inhibitors then used as the feedstock of hydrolysate preparation, glucose concentration increased due to the reduced inhibition on cellulase activity (Table 1). The lipid accumulation increased significantly to 5.93 g/L, 7.37 g/L and 8.05 g/L for the hydrolysates prepared at 15%, 20%, and 25% solids contents, respectively (Fig. 2), while the glucose consumption rate increased

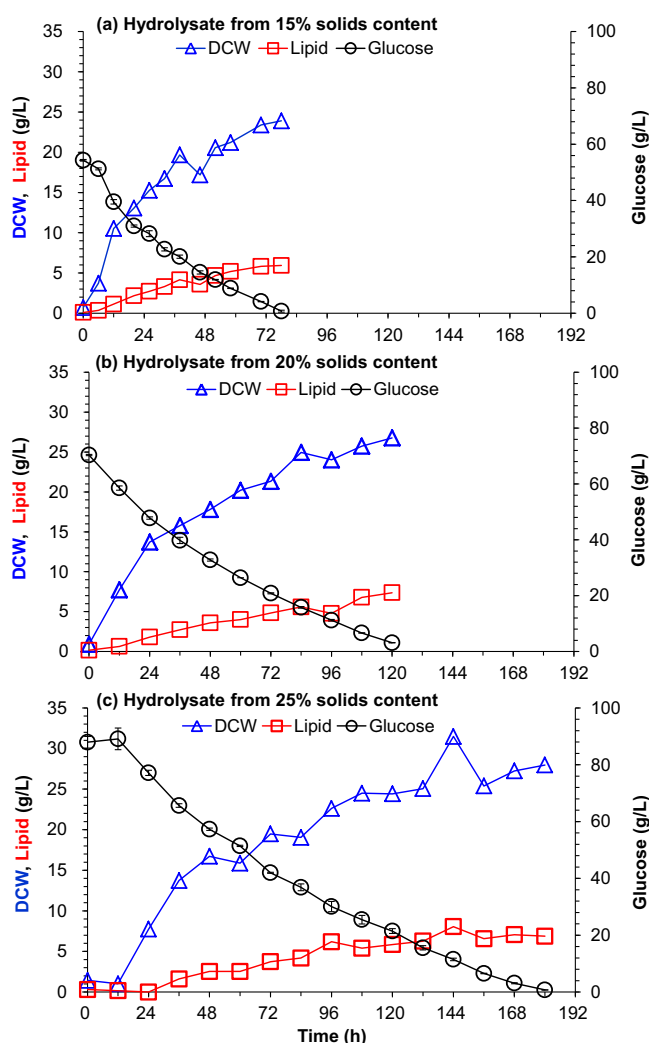
to 0.69 g/(L·h), 0.56 g/(L·h), and 0.48 g/(L·h) from the original 0.55 g/(L·h), 0.39 g/(L·h), and 0.16 g/(L·h), respectively, for each hydrolysate after inhibitor removal by biodetoxification.

The composition of the obtained lipid included oleate ( $C_{18:1}$ , 42.11%), palmitate ( $C_{16:0}$ , 19.41%), palmitoleate ( $C_{16:1}$ , 10.69%), margaroleate ( $C_{17:1}$ , 7.90%), stearate ( $C_{18:0}$ , 5.98%), linoleate ( $C_{18:2}$ , 4.26%), margarate ( $C_{17:0}$ , 3.56%), pentadecanoate ( $C_{15:0}$ , 0.67%), lignocerate ( $C_{24:0}$ , 0.46%), myristate ( $C_{14:0}$ , 0.23%), and nonadecanoate ( $C_{19:0}$ , 0.22%). The lipid was extracted and transesterified with methanol by KOH as described in the Method section. The biodiesel was purified and assayed according to American Society of Testing Materials (ASTM) standard (Table 2). All the properties of the synthesized biodiesel including acid value, copper strip corrosion, kinetic viscosity, flash point and sulfur content well met the ASTM standards expect the water content (0.091%) was slightly higher than the ASTM standard (<0.05%) due to the insufficient drying after the biodiesel synthesis.

### 3.2. Inhibitor tolerance and degradation performance of *T. cutaneum* ACCC 20271

The tolerance comparison of the six typical oleaginous yeast strains including *T. cutaneum*, *L. starkeyi*, *R. glutinis* and *R. toruloides* under the stress of the six typical inhibitor compounds of furfural, HMF, formic acid, levulinic acid, vanillin, 4-hydroxybenzaldehyde show that *T. cutaneum* might be the only surviving oleaginous yeast strain in the relatively high inhibitor containing lignocellulose hydrolysates (Chen et al., 2009; Gao et al., 2014). The detailed inhibitor tolerance and degradation performance of *T. cutaneum* ACCC 20271 were systematically examined under varying concentration of typical inhibitor compounds.

The two furan aldehydes derived from the dehydration of pentose and hexose sugars, furfural and HMF, harshly inhibited the cell growth and lipid accumulation of *T. cutaneum* ACCC 20271. Furfural at only 0.5 g/L reduced the dry cell weight by 65.0% and lipid titer by 90.6% (Fig. 3a). HMF was less toxic compared to furfural, with the reduction of 35.3% of dry cell weight and 24.7% of lipid titer at 5.0 g/L of HMF (Fig. 3b). Acetic, formic and levulinic acids are the most common weak acids present in lignocellulosic hydrolysates. 6 g/L of formic acid only led to reduction of dry cell weight and lipid titer by 17.9% and 25.7% (Fig. 3c). Higher titer of acetic acid at 25 g/L only led to the reduction of 49.6% and 34.0%



**Fig. 2.** Lipid fermentation of *T. cutaneum* ACCC 20271 using corn stover hydrolysates prepared from pretreated corn stover after biodetoxification. (a) hydrolysate prepared at 15% biodetoxified corn stover content; (b) hydrolysate prepared at 20% biodetoxified corn stover content; (c) hydrolysate prepared at 25% biodetoxified corn stover content. Culture conditions: pH at 5.0, 30 °C, 600 rpm, aeration rate 1.67 vvm.

**Table 2**

Assay of biodiesel synthesized using lipid extracted from *T. cutaneum* cells.

Properties	Values	ASTM standard	Assay methods used
Acid value (KOH mg/g)	0.28	<0.8	GB/T264 <sup>a</sup>
Copper strip corrosion (50 °C, 3 h)	1	<3	GB/T5096 <sup>a</sup>
Water content (%)	0.091	<0.05	SH/T0246 <sup>a</sup>
Kinetic viscosity (20 °C, mm <sup>2</sup> /s)	6.42	1.9–6.0 (40 °C)	GB/T265 <sup>a</sup>
Flash point (°C)	178.5	>100	GB/T 261 <sup>a</sup>
Sulfur content (mg/kg)	<0.5	<50	SH/T0689 <sup>a</sup>
Density (g/cm <sup>3</sup> )	0.8809	–	GB/T2540 <sup>a</sup>
Lubrication (HFRR, wear scar diameter, μm)	587	–	MQ-800 four ball testing machine <sup>b</sup>
Condensation point (°C)	Undetected	–	SH/T0248 <sup>a</sup>
Fatty acid methyl ester (%)	>97	–	GC–MS analysis in this study

<sup>a</sup> Assayed according to China Standard for petroleum products.

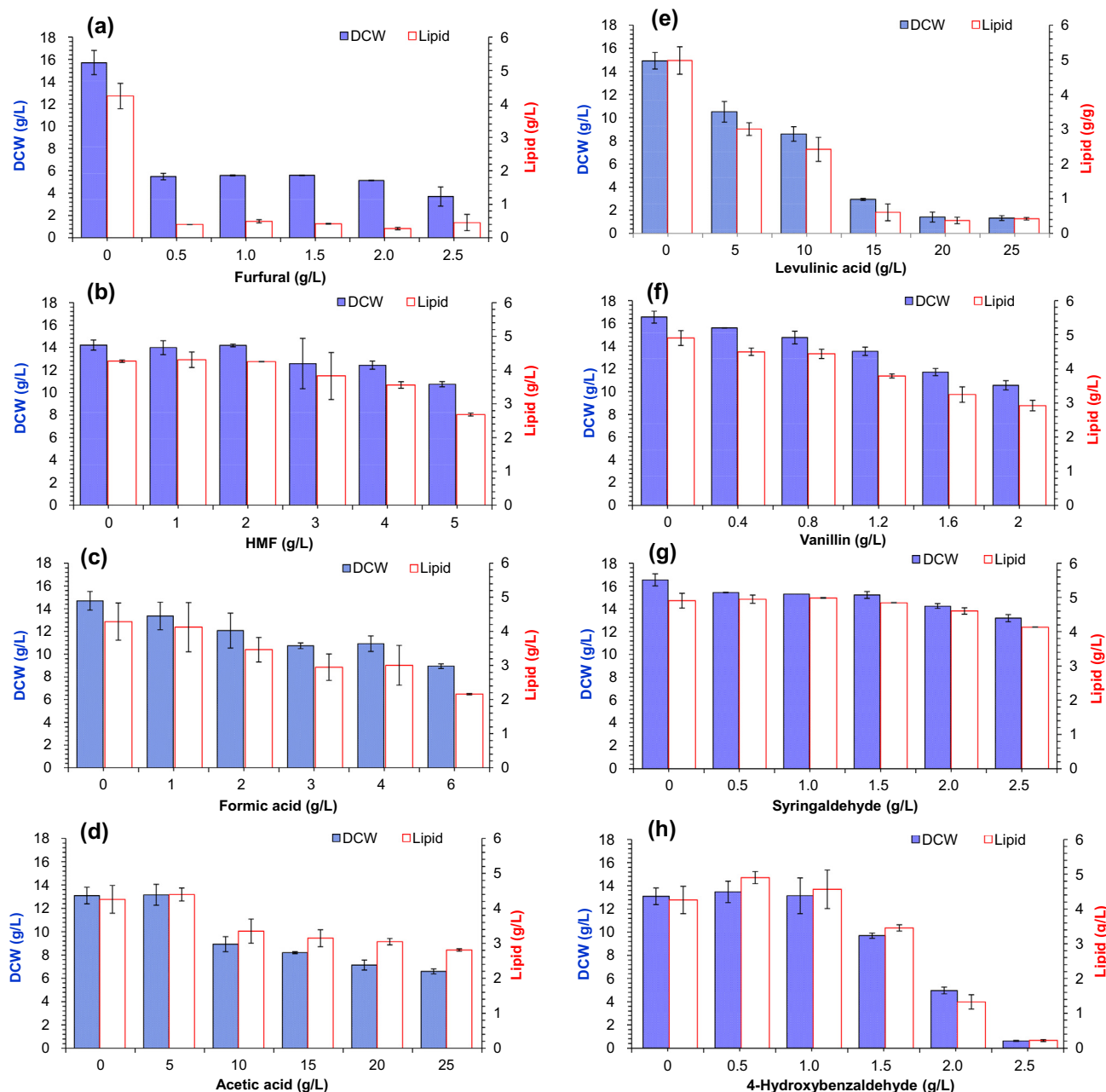
<sup>b</sup> Lubrication was assayed using MQ-800 four ball testing machine (SGW-10G, Jinan Hengxu Machinery, Jinan, Shandong, China) and represented by the wear scar diameter. The rotating speed of the top ball was 1450 rpm, and the radius of balls was 12.7 mm. The grinding spot diameter of three downside balls was measured through optical microscope.



(Fig. 3d), respectively. Levulinic acid at 15 g/L only led to slight reduction of cell growth and lipid titer (Fig. 3e). Three typical phenolic aldehydes, vanillin, syringaldehyde and 4-hydroxybenzaldehyde, were selected to represent the lignin derivatives of guaiacyl group (G), syringyl group (S) and hydroxyphenyl group (H), respectively (Klinke et al., 2004). Syringaldehyde showed only negligible inhibition on cell growth with 2.5 g/L (Fig. 3g). 4-Hydroxybenzaldehyde at 1.5 g/L significantly inhibited the cell growth and at 2.5 g/L almost it completely inhibited the cell growth (Fig. 3h), and vanillin at 2.0 g/L partially inhibited the cell growth (Fig. 3f). Quantitatively, 0.5 g/L of furfural and 2 g/L of 4-hydroxybenzaldehyde led to the half reduction of cell growth of *T. cutaneum* ACCC 20271, while higher HMF at 5 g/L, formic acid at 3 g/L, acetic acid at 10 g/L, levulinic acid at 5 g/L, vanillin at 1.2 g/L syringaldehyde at 2.5 g/L only led to 20% reduction of cell growth. The robustness of *T. cutaneum* ACCC 20271 could be

demonstrated by comparing to other oleaginous yeasts: the cell growth of *R. toruloides* Y4 and *R. toruloides* AS 2.1389 were completely inhibited by 2 g/L of vanillin or 1.22 g/L of 4-hydroxybenzaldehyde, and partially inhibited by 2.19 g/L of syringaldehyde (decreased by 15.7%), 4 g/L of formic acid (decreased by 40%), 10 g/L of acetic acid (decreased by 50%) (Hu et al., 2009; Zhao et al., 2012).

Biodegradation of inhibitors by *T. cutaneum* ACCC 20271 was investigated by adding each inhibitor as the sole carbon source in the medium. The concentration of each inhibitor for biodegradation experiments was selected by referring the corresponding inhibitor level in the pretreated corn stover (He et al., 2016): 1.0 g/L for furfural, 3.0 g/L for HMF, 3.0 g/L for formic acid, 15.0 g/L for acetic acid, 10.0 g/L for levulinic acid, 2.0 g/L for vanillin, 2.5 g/L for syringaldehyde, and 1.0 g/L for 4-hydroxybenzaldehyde, respectively. The furfural and HMF were converted to the corresponding

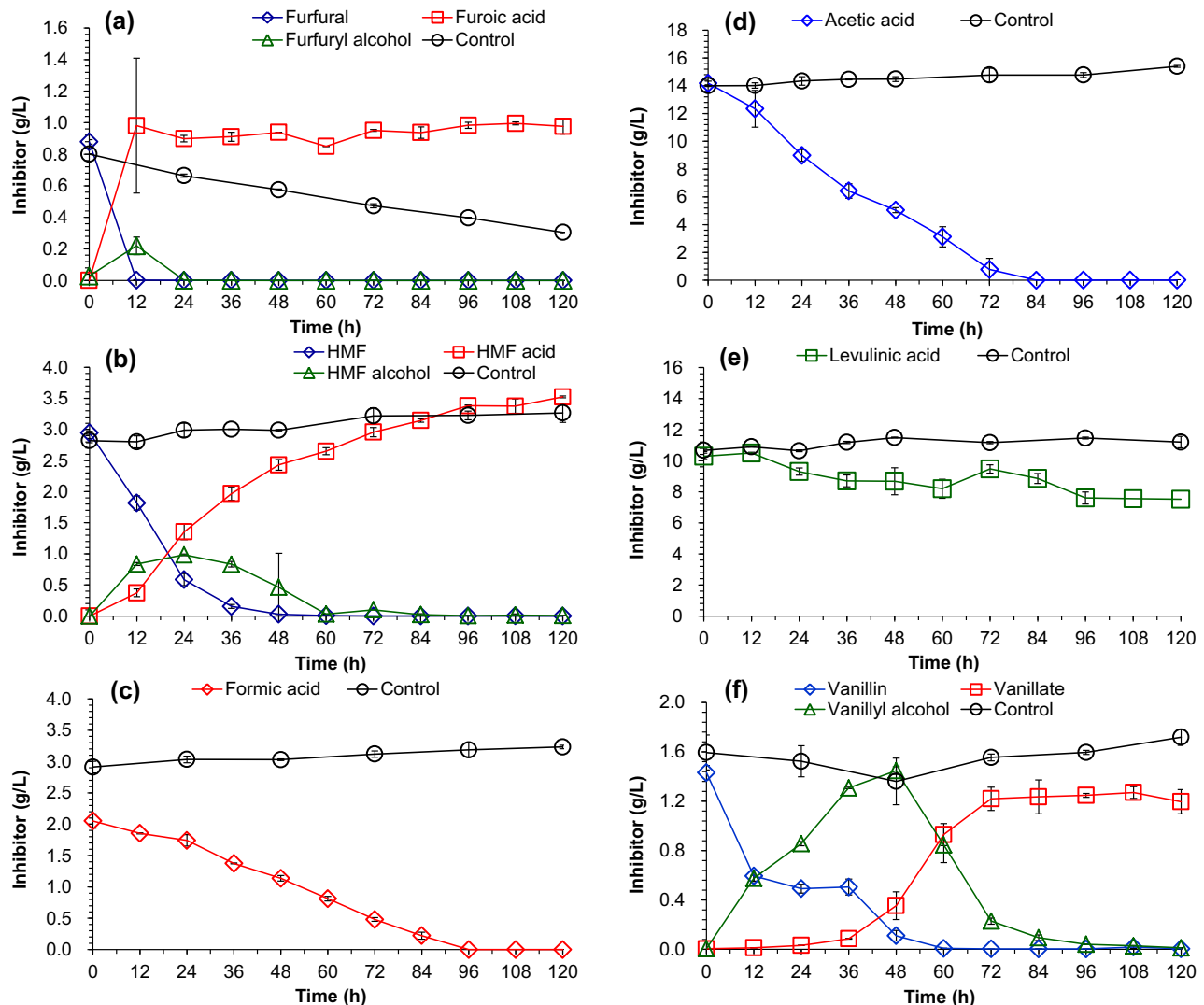


**Fig. 3.** Tolerance of *T. cutaneum* ACCC 20271 to lignocellulose derived inhibitors. (a) furfural (2-furaldehyde); (b) HMF (5-hydroxymethylfurfural); (c) formic acid; (d) acetic acid; (e) levulinic acid; (f) vanillin; (g) syringaldehyde; (h) 4-hydroxybenzaldehyde. Culture conditions: pH at 5.0, 30 °C, 180 rpm for 120 h.

alcohols (furfuryl alcohol and HMF alcohol) within 12 h and 24 h, respectively, then into the corresponding acids (furoic acid and HMF acid), but no further decrease of these acids were observed (Fig. 4a, b). For further confirmation, 1 g/L of furoic acid and 2.5 g/L of HMF acid were separately added, again no degradations of the acids were observed; 2 g/L of furan-2,5-dicarboxylate, the potential metabolite of HMF acid, was also not degraded by *T. cutaneum* ACCC 20271. The results indicate that furoic acid and HMF acid might be the final metabolites of furfural and HMF of *T. cutaneum* ACCC 20271, instead of ultimate degradation by central metabolic pathway. Formic acid and acetic acid were completely degraded by *T. cutaneum* ACCC 20271 after 84 h or 36 h (Figs. 4c, 4d), respectively, while levulinic acid was only partially degraded by *T. cutaneum* ACCC 20271 (Fig. 4e). No relative metabolites were detected for the degradation of these weak acids. Vanillin was degraded within 48 h with the formation of vanillyl alcohol and vanillate, then vanillate slightly decreased with the approximately 24% reduction of the total moles of vanillin, vanillyl alcohol and vanillate after the culture period of 120 h (Fig. 4f). 4-Hydroxybenzaldehyde was degraded into 4-hydroxybenzyl alcohol and 4-hydroxybenzoate within 24 h, then 4-hydroxybenzoate

decreased quickly after its formation (Fig. 4h), indicating that 4-hydroxybenzaldehyde could be ultimately consumed in the consequent central metabolisms. For syringaldehyde, the total moles of syringaldehyde, syringic alcohol and syringate was approximately constant with time (0 h, 13.01 mM; 120 h, 14.08 mM) (Fig. 4g), and no reduction of syringate was observed when it was used as the sole carbon source, indicating that syringaldehyde conversion stopped at its acid form by *T. cutaneum* ACCC 20271, similar with furfural and HMF.

Comparing to oleaginous yeasts *R. toruloides* Y4, *R. toruloides* AS 2.1389, the tolerance of *T. cutaneum* ACCC 20271 has the slightly better or similar tolerance to furfural and HMF, but the tolerance to weak acids (formic acid, acetic acid) and phenolic aldehydes (vanillin, syringaldehyde and 4-hydroxybenzaldehyde) is significantly stronger. The ultimate degradation of formic acid, acetic acid, vanillin and 4-hydroxybenzaldehyde by *T. cutaneum* ACCC 20271, as well as the partial conversion into less toxic alcohols and acids of furfural, HMF and syringate provided the strong supports for its tolerance since the microbial tolerance is generally understood as the biodegradation capability into less toxic derivatives (Liu et al., 2009; Liu and Moon, 2009).



**Fig. 4.** Inhibitors degradation by *T. cutaneum* ACCC 20271. (a) furfural; (b) HMF; (c) formic acid; (d) acetic acid; (e) levulinic acid; (f) vanillin; (g) syringaldehyde; (h) HBA (4-hydroxybenzaldehyde). Culture conditions: pH at 5.0, 30 °C, 180 rpm for 120 h in synthetic medium. Control: inoculated equal volume of water instead of seed culture broth.

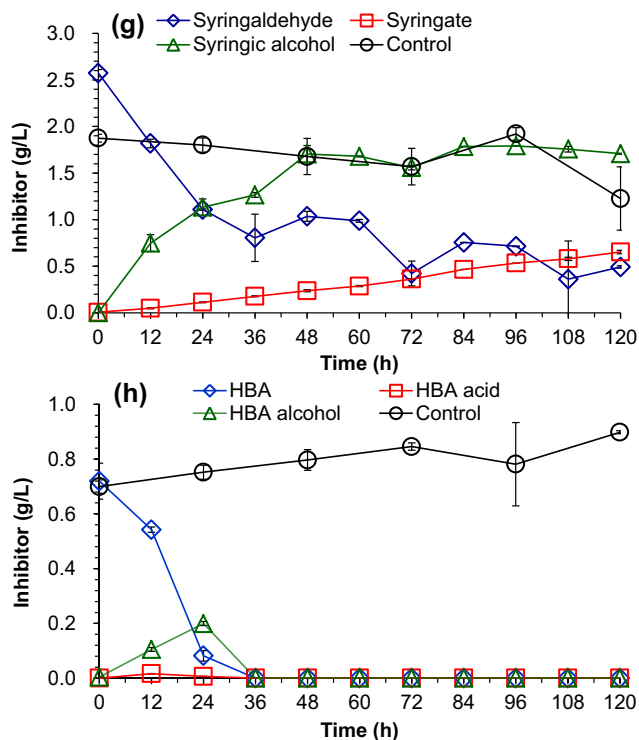


Fig. 4 (continued)

### 3.3. Metabolic pathway analysis on inhibitors conversions of *T. cutaneum* ACCC 20271

Biodegradation pathways of the typical inhibitors of furan aldehydes (furfural and HMF), weak acids (formic acid, acetic acid), and phenolic aldehydes (vanillin, syringaldehyde, 4-hydroxybenzaldehyde) in *T. cutaneum* ACCC 20271 were constructed based on the above experimental results, the genome annotation, and the previous relevant studies (Fig. S1). The larger genome size of *T. cutaneum* ACCC 20271 (LTAL000000000, 30.45 Mb), approximately 50% greater than those of the other three typical oleaginous yeasts, *R. toruloides* NP11 (LNQ000000000.1, 20.38 Mb), *Y. lipolytica* CLIB122 (GCA\_000002525.1, 20.55 Mb), and *T. oleaginosus* IBC0246 (JZUH000000000.1, 19.84 Mb), might have provided more genetic resources for inhibitor degradation capacity of *T. cutaneum* ACCC 20271.

Furfural and HMF are reduced to furfuryl alcohol and HMF alcohol, respectively, by alcohol dehydrogenase (alcohol dehydrogenase (NAD<sup>+</sup>), alcohol dehydrogenase (NADP<sup>+</sup>), short-chain alcohol dehydrogenase, Zn-dependent alcohol dehydrogenases, alcohol dehydrogenase (iron-type) and other alcohol dehydrogenase), aldehyde reductase (aldehyde reductase, semialdehyde reductase, aldo/keto reductase), then the two alcohols are oxidized back to furfural and HMF but at very low concentration, which is not harmful to the growth of *T. cutaneum* cells, by alcohol dehydrogenase or aldehyde reductase. Finally, furfural and HMF are oxidized to furoic acid and HMF acid by aldehyde hydrogenases (aldehyde dehydrogenase (NAD(P)<sup>+</sup>), salicylaldehyde dehydrogenase, D-lactaldehyde dehydrogenase, aminoadipate-semialdehyde dehydrogenase, betaine aldehyde dehydrogenase, semialdehyde dehydrogenase) and the further conversions are stopped in *T. cutaneum* ACCC 20271 according to the experimental results (Fig. S1a).

Vanillin, syringaldehyde and 4-hydroxybenzaldehyde are converted into the corresponding alcohols and then the acids in the similar way to furfural and HMF (Fig. S1c). Firstly, vanillate,

syringaldehyde and 4-hydroxybenzaldehyde are reduced to vanillyl alcohol, syringyl alcohol and 4-hydroxybenzyl alcohol by alcohol dehydrogenase (alcohol dehydrogenase (NAD<sup>+</sup>), alcohol dehydrogenase (NADP<sup>+</sup>), short-chain alcohol dehydrogenase, Zn-dependent alcohol dehydrogenases, alcohol dehydrogenase (iron-type), or other alcohol dehydrogenase), aldehyde reductase (aldehyde reductase, semialdehyde reductase, aldo/keto reductase), then the alcohols are oxidized back to low titer vanillin, syringaldehyde and 4-hydroxybenzaldehyde, then further oxidized into vanillate, syringate and 4-hydroxybenzoate by alcohol dehydrogenase, aldehyde reductase or vanillyl alcohol oxidase (responsible for the conversion of vanillyl alcohol to vanillin). Different from furfural and HMF, the conversion of vanillate and 4-hydroxybenzoate do not stop and further conversion proceeds according to experimental results. The putative intermediate product of the conversions is protocatechuate by catechol 1,2-dioxygenase (CAT, Trcu\_03891, Trcu\_00122) or nitric oxide dioxygenase (Trcu\_05557), which shares the high similarity with vanillate monooxygenase by amino acid sequence blast. Protocatechuate is converted to catechol by 2,3-dihydroxybenzoate decarboxylase (Trcu\_02387) and to *cis*, *cis*-muconate by catechol 1,2-dioxygenase (CAT, Trcu\_03891, Trcu\_00122), and finally converted into succinyl-CoA or acetyl-CoA (Fig. S1d) before it is assimilated into tricarboxylic acid (TCA) cycle for ultimate degradation. On the contrary, experimental results show that syringate is not able to be further degraded and stops at its acid form by *T. cutaneum* ACCC 20271, similar to furfural and HMF.

The encoding genes of alcohol dehydrogenases, aldehyde reductase and aldehyde dehydrogenase responsible for the degradation of furan and phenolic aldehydes, are thoroughly screened and identified in the genome of *T. cutaneum* ACCC 20271 (Table 3). Totally 101 putative genes may play active roles in the conversions of furan aldehydes and phenolic aldehydes in *T. cutaneum* ACCC 20271, including 40 alcohol dehydrogenases (eighteen NAD<sup>+</sup> dependent alcohol dehydrogenases, four NADP<sup>+</sup> dependent alcohol dehydrogenases, five short-chain alcohol dehydrogenases, thirteen Zn-dependent alcohol dehydrogenases), 19 aldehyde reductases (four aldehyde reductases, two semialdehyde reductases, thirteen aldo/keto reductases), 39 aldehyde dehydrogenases (twenty-six aldehyde dehydrogenases, two salicylaldehyde dehydrogenases, two aminoadipate-semialdehyde dehydrogenases, one betaine aldehyde dehydrogenase, eight semialdehyde dehydrogenases) and 3 oxidases (one vanillyl alcohol oxidase, one glucose oxidase, one choline oxidase). Comparing with *T. cutaneum* ACCC 20271, the number of putative functional genes in the genomes of other three typical oleaginous yeasts *R. toruloides* NP11, *Y. lipolytica* CLIB122, and *T. oleaginosus* IBC0246 are significantly small: alcohol dehydrogenases, 28 in *R. toruloides* NP11, 9 in *Y. lipolytica* CLIB122, and 13 in *T. oleaginosus* IBC0246, comparing to 40 in *T. cutaneum* ACCC 20271; aldehyde reductases, 3 in *R. toruloides* NP11, 13 in *Y. lipolytica* CLIB122, 15 in *T. oleaginosus* IBC0246, comparing to 19 in *T. cutaneum* ACCC 20271; aldehyde dehydrogenase, 16, 14, 20, comparing to 39 in *T. cutaneum* ACCC 20271. The rich source of oxidoreductase genes in genome provides the strong support for high degradation capacity and tolerance of *T. cutaneum* ACCC 20271 to inhibitors than these three oleaginous yeasts.

For weak acid inhibitors, formic acid is converted to carbon dioxide by formate dehydrogenase (Trcu\_03671), similar to the formate dehydrogenase gene RHTO\_06042 in *R. toruloides* and CC85DRAFT\_285545 in *T. oleaginosus* (no corresponding gene in *Y. lipolytica*). Acetic acid is converted to acetyl-CoA by acetyl-CoA synthetase (Trcu\_03003, Trcu\_00570, Trcu\_02518), then enters the TCA cycle ultimately (Fig. S1b), similar to acetyl-CoA synthetase gene RHTO\_02900, RHTO\_08027 in *R. toruloides* (Zhu et al., 2012), and YALI0\_F05962g in *Y. lipolytica*

**Table 3**

Putative genes involved in the biodegradation of furan aldehydes and phenolic aldehydes in *T. cutaneum* ACCC 20271, *R. toruloides* NP11, *Y. lipolytica* CLIB122 and *T. oleaginosus* ICB0246.

Encoding enzymes	<i>T. cutaneum</i> ACCC 20271 Numbers and locus		<i>R. toruloides</i> NP11 Numbers and locus		<i>Y. lipolytica</i> CLIB122 Numbers and locus		<i>T. oleaginosus</i> ICB0246 Numbers and locus	
<i>Alcohol dehydrogenases</i>								
Alcohol dehydrogenase (NAD <sup>+</sup> )	18	Trcu_00103, Trcu_00411, Trcu_02229, Trcu_02501, Trcu_04032, Trcu_04134, Trcu_00679, Trcu_00347, Trcu_00109, Trcu_00589, Trcu_00604, Trcu_00714, Trcu_01328, Trcu_02221, Trcu_02684, Trcu_04134, Trcu_04221, Trcu_04303	8	RHTO_00513, RHTO_00517, RHTO_03062, RHTO_03098, RHTO_04685, RHTO_01725, RHTO_02519, RHTO_03798	1	YALIOE17787g	5	CC85DRAFT_301122, CC85DRAFT_286228, CC85DRAFT_288323, CC85DRAFT_285125, CC85DRAFT_165394
Alcohol dehydrogenase (NADP <sup>+</sup> )	4	Trcu_01821, Trcu_04946, Trcu_00053, Trcu_00098	2	RHTO_01922, RHTO_01930,	1	YALIOB16192g		
Short-chain alcohol dehydrogenase	5	Trcu_01507, Trcu_04730, Trcu_04786, Trcu_04816, Trcu_05676	/	/	/	/	/	/
Zn-dependent alcohol dehydrogenases	13	Trcu_00053, Trcu_00098, Trcu_00411, Trcu_02229, Trcu_02501, Trcu_04032, Trcu_04134, Trcu_04221, Trcu_04303, Trcu_04946, Trcu_00103, Trcu_02195, Trcu_01654	9	RHTO_04635, RHTO_07889, RHTO_00661, RHTO_01633, RHTO_03808, RHTO_03847, RHTO_03880, RHTO_04424, RHTO_04424	5	YALIO_D25630g, YALIO_A15147g, YALIO_A16379g, YALIO_E12463g, YALIO_E07766g	3	CC85DRAFT_272173, CC85DRAFT_285852, CC85DRAFT_327221
Alcohol dehydrogenase (iron-type)	/	/	2	RHTO_00263, RHTO_04708	/	/	1	CC85DRAFT_282431
Other alcohol dehydrogenase	/	/	5	RHTO_02673, RHTO_03075, RHTO_06892, RHTO_03559, RHTO_05962	1	YALIO_F09603g,	2	CC85DRAFT_271749, CC85DRAFT_264068
<i>Aldehyde reductases</i>								
Aldehyde reductase	4	Trcu_01328, Trcu_01463, Trcu_01821, Trcu_03118	2	RHTO_03964, RHTO_03032	/	/	2	CC85DRAFT_300021, CC85DRAFT_249059
Semialdehyde reductase	2	Trcu_01507, Trcu_05676	/	/	/	/	/	/
Aldo/keto reductase	13	Trcu_03868, Trcu_00463, Trcu_00843, Trcu_01328, Trcu_01410, Trcu_01463, Trcu_01821, Trcu_01822, Trcu_01943, Trcu_02999, Trcu_03519, Trcu_03746, Trcu_05608, Trcu_01943	3	RHTO_00641, RHTO_06555, RHTO_03963	13	YALIO_D07634g, YALIO_F18590g, YALIO_A15906g, YALIO_E18348g, YALIO_B07117g, YALIO_A19910g, YALIO_C00319g, YALIO_D04092g, YALIO_B21780g, YALIO_C09119g, YALIO_F06974g, YALIO_C13508g, YALIO_B15268g	15	CC85DRAFT_282180, CC85DRAFT_151847, CC85DRAFT_284302, CC85DRAFT_284949, CC85DRAFT_318912, CC85DRAFT_285592, CC85DRAFT_285664, CC85DRAFT_232176, CC85DRAFT_247936, CC85DRAFT_262565, CC85DRAFT_329428, CC85DRAFT_147886, CC85DRAFT_287572, CC85DRAFT_264235, CC85DRAFT_287865
<i>Aldehyde dehydrogenases</i>								
Aldehyde dehydrogenase (NAD (P) <sup>+</sup> )	26	Trcu_03118, Trcu_01234, Trcu_01775, Trcu_02638, Trcu_00064, Trcu_00068, Trcu_01234, Trcu_01775, Trcu_02569, Trcu_02638, Trcu_02661, Trcu_03118, Trcu_03765, Trcu_03855, Trcu_03870, Trcu_04064, Trcu_04141, Trcu_04359, Trcu_04385, Trcu_04408, Trcu_05408, Trcu_05469, Trcu_05623, Trcu_04141, Trcu_00404, Trcu_05610	10	RHTO_00119, RHTO_06090, RHTO_06724, RHTO_01654, RHTO_03710, RHTO_04310, RHTO_04425, RHTO_04543, RHTO_05680, RHTO_05838	14	YALIO_F23793g, YALIO_E15400g, YALIO_B01298g, YALIO_A17875g, YALIO_F26191g, YALIO_B09647g, YALIO_C01859g, YALIO_E30481g, YALIO_E11341g, YALIO_A19448g, YALIO_C03025g, YALIO_E00264g, YALIO_F04444g, YALIO_D07942g	14	CC85DRAFT_294805, CC85DRAFT_283248, CC85DRAFT_256663, CC85DRAFT_283984, CC85DRAFT_284080, CC85DRAFT_272549, CC85DRAFT_259523, CC85DRAFT_302667, CC85DRAFT_329114, CC85DRAFT_277397, CC85DRAFT_287487, CC85DRAFT_287882, CC85DRAFT_287961, CC85DRAFT_200477, CC85DRAFT_283363, CC85DRAFT_272464, CC85DRAFT_327950, CC85DRAFT_263114, CC85DRAFT_330956, CC85DRAFT_288983
Salicylaldehyde dehydrogenase	2	Trcu_03855, Trcu_04531	5	RHTO_00385, RHTO_06811, RHTO_01494, RHTO_02062, RHTO_05993			6	

(continued on next page)



Table 3 (continued)

Encoding enzymes	<i>T. cutaneum</i> ACCC 20271 Numbers and locus		<i>R. toruloides</i> NP11 Numbers and locus		<i>Y. lipolytica</i> CLIB122 Numbers and locus		<i>T. oleaginosus</i> ICB0246 Numbers and locus	
D-lactaldehyde dehydrogenase	/	/	1	RHTO_03406	/	/	/	/
Aminoadipate-semialdehyde dehydrogenase	2	Trcu_05610, Trcu_01477	/	/	/	/	/	/
Betaine aldehyde dehydrogenase	1	Trcu_04408	/	/	/	/	/	/
Semialdehyde dehydrogenase	8	Trcu_00064, Trcu_00404, Trcu_04064, Trcu_04737, Trcu_05408, Trcu_05469, Trcu_05543, Trcu_02661	/	/	/	/	/	/
<i>Oxidases</i>								
Alcohol oxidase	/	/	2	RHTO_01165, RHTO_02441	1	YALI0_B14014g	2	CC85DRAFT_317844, CC85DRAFT_233638
Vanillyl alcohol oxidase	1	Trcu_03959	2	RHTO_01165, RHTO_02441	/	/	/	/
Glucose oxidase	1	Trcu_01554	/	/	/	/	/	/
Choline oxidase	1	Trcu_04415	1	RHTO_06508	/	/	1	CC85DRAFT_283985

(Dujon et al., 2004), and CC85DRAFT\_283253, CC85DRAFT\_259369, CC85DRAFT\_261919 in *T. oleaginosus* (Kourist et al., 2015).

#### 4. Conclusions

Lipid accumulation of *T. cutaneum* ACCC 20271 using lignocellulose feedstock and its potential on biodiesel synthesis are evaluated. The tolerance and biodegradation of *T. cutaneum* ACCC 20271 on the lignocellulose derived inhibitors was experimentally and systematically investigated. The degradation pathways of the inhibitors in *T. cutaneum* ACCC 20271 was proposed based on the experimental results, the genome annotation, and relevant studies. This study provides the first insight on the lipid production potential and inhibitor degradation pathway analysis of *T. cutaneum* for the future application of cellulosic lipid production.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2016.06.130>.

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