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Screening of Oleaginous Yeast Strains Tolerant to Lignocellulose Degradation Compounds

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Abstract High cost of triacylglycerol lipid feedstock is the major barrier for commercial production of biodiesel. The fermentation of oleaginous yeasts for lipid production using lignocellulose biomass provides a practical option with high economic competitiveness. In this paper, the typical oleaginous yeast strains were screened under the pressure of lignocellulose degradation compounds for selection of the optimal strains tolerant to lignocellulose. The inhibitory effect of lignocellulose degradation products on the oleaginous yeast fermentation was carefully investigated. Preliminary screening was carried out in the minimum nutritious medium without adding any expensive complex ingredients then was carried out in the lignocellulosic hydrolysate pretreated by dilute sulfuric acid. Seven typical lignocellulose degradation products formed in various pretreatment and hydrolysis processing were selected as the model inhibitors, including three organic acids, two furan compounds, and two phenol derivatives. The inhibition of the degradation compounds on the cell growth and lipid productivity of the selected oleaginous yeasts were examined. Acetic acid, formic acid, furfural, and vanillin were found to be the strong inhibitors for the fermentation of oleaginous yeasts, while levulinic acid, 5-hydroxymethylfurfural, and hydroxybenzaldehyde were relatively weak inhibitors. *Trichosporon cutaneum* 2.1374 was found to be the most adopted strain to the lignocellulose degradation compounds.

Keywords Triacylglycerol lipid · Oleaginous yeasts · *Trichosporon cutaneum* · Screening · Lignocellulose degradation compounds

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Introduction

Biodiesel is a diesel-equivalent fatty acid alkyl esters produced through transesterification reaction of triacylglycerol lipids with methanol or ethanol. It is an ideal substitute of the conventional vehicles diesel for its biodegradable, nontoxic, and typically produces about 60% less net carbon dioxide emissions than petroleum-based diesel. Generally, the cost of the triacylglycerol such as vegetable oils, animal fats, or recycled greases and other oil resources is about 70–75% of the total biodiesel production [1, 2]. The meaningful replacement of biodiesel to petroleum based diesel on the transportation fuel market requires the sustainable and stable supply of cheap triacylglycerol lipid to release the pressure on the ever-increasing prices of soybean oil and rapeseed oils [3–5]. Two resolutions have been exploited, the first one is to grow oil plants in the dry, mountain, or saline lands; the second one is through the fermentation of oleaginous microorganisms in the bioreactor [6]. Oleaginous microorganisms capable of metabolizing lignocellulose-derived sugars could provide a practical option with high economic competitiveness [7]. The key technical barrier to this goal is the high of fermentation process and one of the major resolution is using cheap fermentation medium, such as fermentation waste fluents, lignocellulose hydrolysate without the addition of expensive fermentation ingredients such as yeast extract, peptone, etc.

Lignocellulose such as agriculture stover, forest residues, and energy plants is the most abundant and sustainable biomass on earth with low commercial values currently [8]. The cost for production of triacylglycerol lipid could be considerably reduced if lignocellulosic biomass is used as carbon source. To obtain the fermentable sugars from the lignocellulose, a harsh physical or chemical process called pretreatment such as steam explosion or dilute acid pretreatment is applied to break the lignin or hemicellulose shell and transform the crystalline cellulose to amorphous cellulose. Various degradation compounds are released from lignocellulose degradation during the pretreatment and will enter the downstream hydrolysis and fermentation processes. These degradation products in the hydrolysate include sugar degradation products such as furfural from xylose and 5-hydroxymethylfurfural (5-HMF) from glucose [9]; lignin degradation products such as vanillin, syringaldehyde, and 4-hydroxybenzaldehyde [10]; organic acids such as acetic acid from acetyl group, formic acid from xylose oxidation, and levulinic acid from glucose oxidation [11]. The composition of the degradation products depends on the type of lignocellulosic materials used, as well as the chemistry and the nature of the pretreatment process. It has been well realized and carefully investigated that the lignocellulose degradation products are strong inhibitors to ethanol fermentation [12]. Up to now, very little knowledge was known about the fermentation performance and bio-lipid accumulation of oleaginous microorganisms under the stress and tolerance of lignocellulose degradation compounds.

Oleaginous microorganisms such as yeast, mould, bacterium, and algae can be used to produce a great deal of lipid, up to 70% lipid of the total dry cell weight under the certain conditions [6, 7, 13]. Among these microorganisms, the eucaryotic yeasts, fungi, and algae synthesize polyunsaturated fatty acid triacylglycerol similar to vegetable oil, while the prokaryotic bacterium synthesizes special lipids. In this work, oleaginous yeasts were chosen as the fermentation strains for production of lipid using corn stover hydrolysate as the carbon source. The common oleaginous yeasts are including: *Rhodotorula glutinis*, *Trichosporon cutaneum*, *Rhodotorula rubra*, *Rhodosporidium toruloides*, *Lipomyces starkeyi*, *Cryptococcus albidum* [14]. Most of the oleaginous yeasts were covered in this study. The basis principles for screening include: (1) the fatty acid triacylglycerol produced by the oleaginous yeasts meets the requirement as biodiesel feedstock, that is, the carbon

number of the major fatty acid is C16–18. (2) High growth rates on wide varieties of substrates and utilize cheap raw materials. The purpose of this work is to screen the most adopted oleaginous strains tolerant to the lignocellulose degradation compounds based on their inhibitory performance. The tolerance and stress of the degradation inhibitors on the sugar utilization of lignocellulose-derived glucose and xylose, the cell growth, and lipid accumulation properties were quantitatively studied. The results show that the fermentation performance under the tolerance of degradation compounds varies significantly depending on the oleaginous yeast species, inhibitor types, and fermentation conditions. A best inhibitor tolerant strain, *T. cutaneum* 2.1374, was screened and fermented in the lignocellulose hydrolysate with the minimum nutrients for production of triglycerol lipid.

Materials and Methods

Strains and Media

All oleaginous yeast strains used in the experiments were listed in Table 1. The strains were purchased from China General Microbiological Culture Collection Center, Beijing, China. The strains were transferred and maintained on YPD-agar plates containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar–agar. The inoculum medium contains 20 g/L glucose; 0.5 g/L yeast extract; 5 g/L $(NH_4)_2SO_4$; 1 g/L KH_2PO_4; 0.5 g/L $MgSO_4{\cdot}7H_2O$. The fermentation medium is nitrogen-limited which contains only minimum nutrients without the addition of any complex ingredients such as yeast extract, peptone, etc. The composition of the nitrogen-limited or minimum nutrition was fixed in all the culture experiments to 30 g/L glucose; 20 g/L xylose; 2 g/L $(NH_4)_2SO_4$; 1 g/L KH_2PO_4; 0.5 g/L $MgSO_4{\cdot}7H_2O$, unless mentioned otherwise.

Reagents

Furfural and 5-HMF were from Acros Organics (New Jersey, USA). Levulinic acid was from Alfa Aesar (Ward Hill, MA, USA). Vanillin, 4-hydroxybenzaldehyde, sodium hydroxide, sodium acetate were all from Sinopharm Chemical Reagent (Shanghai, China). Glucose, formic acid, acetic acid, and ammonia sulfate were purchased from other local

Table 1 Oleaginous microorganisms used.

Microorganisms	CGMCC ID	Short names
Rhodotorula glutinis	2.107	*R. glutinis* 2.107
Rhodotorula glutinis	2.703	*R. glutinis* 2.703
Rhodotorula glutinis	2.704	*R. glutinis* 2.704
Trichosporon cutaneum	2.571	*T. cutaneum* 2.571
Trichosporon cutaneum	2.1374	*T. cutaneum* 2.1374
Rhodotorula rubra	2.1515	*R. rubra* 2.1515
Rhodosporidium toruloides	2.1389	*R. toruloides* 2.1389
Rhodosporidium toruloides	2.1609	*R. toruloides* 2.1609
Lipomyces starkeyi	2.1390	*L. starkeyi* 2.1390
Lipomyces starkeyi	2.1608	*L. starkeyi* 2.1608

All strains were from CGMCC

CGMCC China General Microbiological Culture Collection Center

chemical reagent companies in Shanghai, China. Two industrial cellulase enzymes used were Spezyme CP from Genencor International (Rochester, NY, USA), and Novozyme 188 from Novo Industrial A/S (purchased from Sigma–Aldrich, St Louis, MO, USA).

Production of Corn Stover Hydrolysate

Corn stover was harvested in fall 2006 from Northeast Province Jilin, China. The corn stover was milled and fractionated though a sieve with the pore diameter of 5 mm. The chipped corn stover was presoaked with 2.5% sulfuric acid for 1.5 h at ambient temperature. The hot steam (3 MPa) was jetted into the 2.5 L pretreatment reactor and kept jetting for 5 min at 180 °C. The pretreated corn stover was separated into a solid fraction and a liquid prehydrolysate. The prehydrolysate was over-limed using calcium hydroxide to pH 5 at 50 °C for 30 min. Then, the over-limed prehydrolysate was mixed with the pretreated solid then to the enzymatic hydrolysis step for production of hydrolysate used in the fermentation. Spezyme CP and Novozyme 188 were added at the amount of 7 FPU and 15 IU/g of dried solid fraction, respectively, in the enzymatic hydrolysis process. The hydrolysis was carried out at 50 °C for 48 h. The water insoluble solid in the hydrolysate was separated by centrifugation and the liquid hydrolysate was used to culture the oleaginous yeasts. The major compositions in lignocellulosic hydrolysate include glucose 84.65 g/L, xylose 36.39 g/L, acetic acid 11.58 g/L, levulinic acid 1.49 g/L, formic acid 2.56 g/L, furfural 0.32 g/L, 5-HMF 1.01 g/L, vanillin 0.061 g/L, and hydroxybenzaldehyde 0.103 g/L.

Culture Conditions and Lipid Recovery

A large single colony of the selected oleaginous yeast strains was picked up from YPD-agar plates and inoculated into 20 mL of inoculum medium for 24 h for the seeding culture; 5-mL seed suspension was inoculated into the 250-mL conical flasks containing 50 mL of fermentation medium in which the nitrogen was regulated by ammonia sulfate addition. The pH of all media was adjusted to 5 with 2 M NaOH and autoclaved at 115 °C for 20 min. All cultures were incubated at an orbit agitation rate of 180 rpm and the temperature of 30 °C. The concentration of sugars and degradation compounds, the dry cell mass, and the lipid composition were sampled periodically. The yeast cells were harvested by centrifugation at 10,000 rpm for 5 min and then washed with deionized water twice to remove the extracellular fat from the cell surface and superfluous nutrient substances. The dry cell mass (DCM) was determined after the cell was dried at 105 °C for 24 h. One gram of the wet cells was disrupted and homogenized in 6 mL of 4 M hydrochloric acid for 30 min. The cell debris was socked for 30 min, heated at 100 °C for 10 min, and then quenched at −20 °C. Frozen cells were stirred with 10 mL methanol–chloroform mixture (methanol–chloroform=1:2 by volume ratio) for 30 min. The extracted lipid was centrifuged to give a clear organic solvent and anhydrous sodium sulfate was added to remove any residual moisture [15]. The solvent was removed by evaporation and the total lipid was measured by gravimetric method.

Analysis of Sugars, Inhibitors, and Lipids

Glucose, xylose, and lignocellulose degradation compounds were analyzed by high performance liquid chromatography (LC-20AD, refractive index detector RID-10A, Shimadzu, Japan) with a Bio-rad Aminex HPX-87H column at the column temperature

65 °C. The mobile phase was 0.005 M H_2SO_4 at the rate of 0.6 mL/min. All samples were centrifuged to remove the cell mass and other water insoluble substances, and then filtered through a 0.22-μm filter before the analysis.

The fatty acid composition of the lipid was analyzed using gas chromatography-mass spectrometry (GC-MS). The Clarus500 gas chromatograph (PerkinElmer) used a PE-5 column (30 m×0.25 mm×0.25 μm). The carrier gas was He at the rate of 1 mL/min; the initial oven temperature was 80 °C, increased at the rate of 16 °C/min, and the final temperature reached 280 °C. First, the lipid was transesterified with methanol into its fatty acid methyl ester form using 10% boron trifluoride as catalytic agent; then the ester was heated to 60 °C for 10 min; finally, it was injected into the GC-MS. The fatty acid composition was found using NIST MS Search 2.0.

Results

Preliminary Screening of Oleaginous Yeasts in the Minimum Nutritious Medium

The cell survival and metabolism in the lignocellulose hydrolysate environment are the first priority during the fermentation. The screening was carried out in the minimum nutritious medium under the nitrogen-limited condition. The medium was prepared with glucose and xylose as the sole carbon sources, ammonia sulfate as the sole nitrogen source, without adding any expensive ingredients such as yeast extract, peptone, and other complex substances. Six oleaginous yeast strains including *T. cutaneum* 2.1374, *R. toruloides* 2.1389, *L. starkeyi* 2.1390, *L. starkeyi* 2.1608, *R. glutinis* 2.704, and *R. glutinis* 2.107 were found to grow well in the designed medium, and the growth profile was shown in Fig. 1. The growth of the rest four strains including *R. glutinis* 2.703, *T. cutaneum* 2.571, *R. rubra* 2.1515, and *R. toruloides* 2.1609 was not observed, indicating that the growth activity of the four strains were weak in the nitrogen-limiting or minimum nutritious medium.

The DCM, the lipid in medium (g/L), and the lipid in cells (wt.%) of these six oleaginous yeast strains after cultured 96 h was shown in Table 2. The lipid in medium was ranged from 0.52 to 2.29 g/L, and the lipid in cells was ranged from 13.0% to 39.8%. The fatty acid chains on the yeast lipid produced was characterized using GC-MS, and the fatty

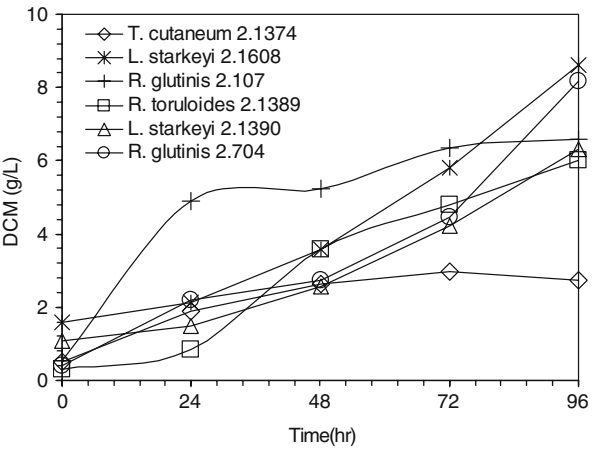

Fig. 1 The growth of six yeasts in the nitrogen-limited medium versus fermentation time. The nitrogen-limited medium including 50 g/L glucose; 2 g/L $(NH_4)_2SO_4$; 1 g/L KH_2PO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$. All cultures were incubated in an orbital shaker at an agitation rate of 180 rpm and incubation temperature 30 °C

Table 2 The DCM, lipid in medium, lipid in cells and lipid yield of the six oleaginous yeast strains after cultured 96 h in the nitrogen-limited medium.

Microorganisms	DCM (g/L)	Lipid in medium (g/L)	Lipid in cells (wt.%)	Lipid productivity (g/L h^{-1})	Lipid yield (g/100 g glucose)
T.cutaneum 2.1374	2.75	1.09	39.8	0.011	10.1
L. starkeyi 2.1608	9.35	2.04	21.8	0.097	10.5
L. starkeyi 2.1390	6.16	2.29	37.2	0.021	10.6
R. glutinis 2.107	4.01	0.52	13.0	0.042	4.92
R. glutinis 2.704	5.49	0.92	16.7	0.057	2.78
R. toruloides 2.1389	4.26	1.67	39.3	0.044	12.6

acid composition showed that the major fatty acids were palmitic acid, stearic acid, oleinic acid, linoleic acid, and palmitoleic acid which are all C16 and C18 fatty acids, either saturated or unsaturated, which were the suitable feedstocks for production of biodiesel.

Screening of the Oleaginous Yeasts Under the Inhibition of Organic Acids

Acetic acid, formic acid, and levulinic acid are the major organic acid components in the lignocellulose hydrolysate. Acetic acid is formed during the hydrolysis of hemicellulose in which the acetyl group of hemicellulose linked to the lignin or cellulose is released and hydrolyzed to form acetic acid. Levulinic acid is the terminal product of oxidation of D-glucose and D-mannose. There are two formation pathways for formic acid formation, one is the terminal product of xylose oxidation and another one is the byproduct of D-glucose and D-mannose oxidation to levulinic acid, both occurred mainly during the chemical pretreatment step [16, 17]. These three compounds were selected to be the representative inhibitors to investigate the inhibitory performance of organic acids released from lignocellulose on the six prescreened oleaginous yeast fermentation.

The effect of acetic acid on the cell culture of *T. cutaneum* 2.1374 was shown in Fig. 2. The growth of all six strains except *T. cutaneum* 2.1374 ceased completely when 5 g/L acetic acid was added to the nitrogen-limited or minimum nutritious medium. For *T.*

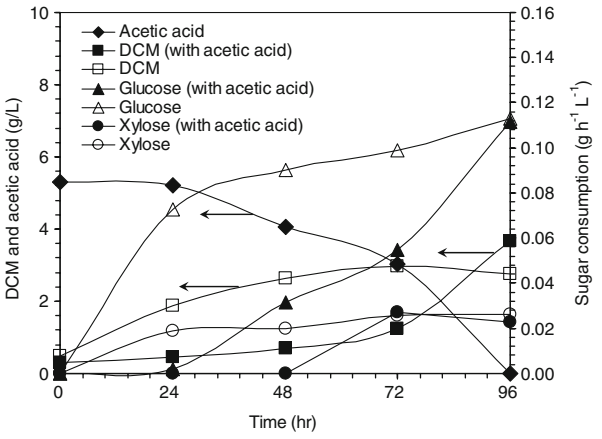

Fig. 2 Time course of the *T. cutaneum* 2.1374 fermentation under the acetic acid inhibition. The medium includes 30 g/L glucose, 20 g/L xylose, 5 g/L acetic acid, 2 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$. All cultures were incubated in an orbital shaker at an agitation rate of 180 rpm and incubation temperature 30 °C

cutaneum 2.1374, the cell growth (indicated by DCM) and the glucose or xylose consumption rate decreased considerably when 5 g/L of acetic acid was added to the medium. Figure 2 also indicated that *T. cutaneum* 2.1374 was able to metabolize acetic acid slowly simultaneously with the glucose or xylose consumption. Probably this was at least the partial reason of the high acetic acid tolerance of *T. cutaneum* 2.1374. At the end of the 96-h cultivation, the maximum cell mass (3.68 g/L) was greater than that without acetic acid addition (2.75 g/L). The lipid productivity with the acetic acid addition (1.12 g/L) was also bit greater than that without acetic acid addition (1.09 g/L). The phenomenon indicated that acetic acid participated the lipid fermentation for production of lipid under the range of *T. cutaneum* 2.1374.

Similar to acetic acid, the growth of all six selected strains were strongly inhibited under the existence of formic acid with the only exception of *T. cutaneum* 2.1374. The result was shown in Table 3. When the concentration of formic acid reached to 3 g/L, the growth of *L. starkeyi* 2.1390, *L. starkeyi* 2.1608 and *R. glutinis* 2.107 completely ceased, and of course there were almost no lipid produced. For *R. glutinis* 2.704 and *R. toruloides* 2.1389, there were only a little growth with almost no lipid production. All these five strains completely ceased to grow when the concentration of formic acid reached to 5 g/L. *T. cutaneum* 2.1374 showed the best tolerance to formic acid, which was almost not affected by formic acid addition. The growth of *T. cutaneum* 2.1374 was even enhanced when the formic acid concentration was 1 g/L at which the maximum DCM was 4.85 g/L.

The inhibition effect of levulinic acid on the selected oleaginous yeast culture was different with that of acetic acid and formic acid. As shown in Table 3, all six strains were not significantly affected by the existence of levulinic acid under the experimental range up to 10 g/L, which was twice as high as the acetic and formic acids tested. On the contrary, the growth of some strains such as *T. cutaneum* 2.1374 was enhanced by the existence of levulinic acid. The maximum dry cell mass and lipid for *T. cutaneum* 2.1374 were 4.34 and 1.19 g/L, relatively, which appeared in the levulinic acid concentration of 10 g/L. For the other five strains, the growth and sugar consumption decreased but not ceased to grow with the increasing concentration of levulinic acid. For *R. toruloides* 2.1389, the maximum DCM and lipid were 4.60 and 1.40 g/L, relatively, which appeared in the levulinic acid concentration of 5 g/L (the data was not showed).

Screening of Oleaginous Yeasts Under the Inhibition of Furan Derivatives from Sugar Degradation

Furfural and 5-HMF are the two major degradation products from xylose and glucose, respectively, and have been well-recognized as the major fermentation inhibitors on the ethanol fermentation. The inhibition effect of these two furan compounds on the lipid fermentation was investigated using the minimum nutritious medium under nitrogen-limited conditions. The results also showed in Table 3. Table 3 showed that furfural strongly inhibited the cell growth and lipid production of all the selected six oleaginous yeast strains under the experimental range. *T. cutaneum* 2.1374 showed the relatively better tolerance to furfural, but the cell almost ceased to grow at the furfural concentration greater than 2 g/L. The maximum DCM was only 2.94 at 0.5 g/L of furfural and the lipid seemed increase little. *R. glutinis* 2.107 and *L. starkeyi* 2.1390 grew only at the minimum furfural (less than 1 g/L); *L. starkeyi* 2.1608, *R. glutinis* 2.704, and *R. toruloides* 2.1389 ceased the growth even at the minimum concentration in the experimental range (0.5 g/L).

5-HMF showed a significant different inhibition performance comparing to furfural. Table 3 showed that 5-HMF inhibited the cell growth and lipid production weakly in the

Table 3 Performance of six strains under the inhibition of six inhibitors in the nitrogen-limited medium.

Microorganisms	Inhibitor concentration (g/L)		DCM (g/L)	Lipid in medium (g/L)	Lipid in cells (wt.%)	Lipid yield (g/100 g glucose)
T. cutaneum 2.1374	Formic acid	1.0	4.85	1.04	21.5	6.00
		3.0	3.58	0.63	17.5	5.30
		5.0	3.02	0.60	20.0	6.30
	Levulinic acid	1.0	2.88	0.79	27.5	6.00
		5.0	4.20	1.19	28.3	6.30
		10	4.34	1.08	25.0	5.40
	Furfural	0.5	2.94	1.25	42.5	12.3
		1.0	1.76	0.54	30.6	6.76
	5-HMF	0.5	1.78	0.83	46.8	10.1
		1.0	2.58	1.14	44.2	12.0
		2.0	2.37	1.04	43.8	10.1
	Vanillin	0.5	3.39	1.24	36.6	10.4
		2.0	2.50	0.98	39.2	10.2
	Hydroxybenzaldehyde	0.5	2.30	0.78	34.0	7.98
		1.5	1.32	0.48	36.5	5.64
L. starkeyi 2.1608	Formic acid	1.0	6.31	0.98	15.5	10.2
	Levulinic acid	1	7.93	0.74	9.33	5.22
		10	2.98	0.24	8.05	5.05
	Furfural	0	9.35	2.04	21.8	10.5
	5-HMF	0.5	9.50	2.00	23.8	11.2
		1.0	8.44	2.08	24.6	11.9
	Vanillin	0.5	7.60	1.58	20.8	9.62
	Hydroxybenzaldehyde	0.5	10.6	2.93	27.7	11.1
L. starkeyi 2.1390	Formic acid	1.0	0.27	0.078	28.9	5.61
	Levulinic acid	1	2.99	0.48	16.1	4.41
		5	2.90	0.30	10.3	2.89
	Furfural	0.5	5.41	1.64	30.3	9.26
	5-HMF	0.5	7.09	2.22	31.3	8.95
		1.0	5.51	2.00	36.3	6.69
	Vanillin	0	6.16	2.29	37.2	10.6
	Hydroxybenzaldehyde	0.5	6.00	2.30	38.3	10.3
R. glutinis 2.704	Formic acid	1	2.13	0.06	2.82	0.69
		3	1.23	0.04	3.25	0.69
	Levulinic acid	1	4.13	0.52	12.6	3.44
		5	4.36	0.44	10.1	2.87
		10	3.91	0.34	8.70	2.86
	Furfural	0	7.42	0.68	9.16	2.78
	5-HMF	0.5	5.29	0.34	6.43	2.03
		1.0	3.86	0.24	6.22	1.96
		2.0	3.12	0.14	4.49	1.40
	Vanillin	0.5	2.96	0.46	15.5	4.40
		1.0	1.65	0.27	16.4	6.03
	Hydroxybenzaldehyde	0.5	5.01	0.30	5.99	2.41
R. glutinis 2.107	Formic acid	1	2.38	0.12	5.04	2.28
	Levulinic acid	1	4.91	0.40	8.15	3.02
		5	5.12	0.36	7.03	2.27
		10	3.56	0.26	7.30	2.30
	Furfural	0.5	3.63	0.20	5.51	2.05

Table 3 (continued)

Microorganisms	Inhibitor concentration (g/L)		DCM (g/L)	Lipid in medium (g/L)	Lipid in cells (wt.%)	Lipid yield (g/100 g glucose)
	5-HMF	0.5	5.11	0.56	11.0	4.27
		1.0	3.05	0.20	6.56	2.12
		2.0	2.93	0.24	8.19	2.31
	Vanillin	0.5	3.58	0.30	8.38	3.21
	Hydroxybenzaldehyde	0.5	3.44	0.38	11.0	3.65
R. toruloides 2.1389	Formic acid	1	2.71	0.20	7.38	1.92
		3	1.73	0.16	9.25	2.17
	Levulinic acid	1	4.2	1.38	32.9	7.75
		5	4.60	1.40	30.4	5.92
		10	3.83	0.73	19.1	4.11
	Furfural	0	4.26	1.67	39.2	12.6
	5-HMF	0.5	3.39	0.76	22.4	6.2
		1.0	2.42	0.40	16.5	4.02
		2.0	1.90	0.28	14.7	3.25
	Vanillin	0.5	1.92	0.36	18.8	4.46
	Hydroxybenzaldehyde	0.5	2.21	0.34	15.4	3.82
		1.0	5.93	1.68	28.3	8.15

Under the inhibitor concentration above the list value, the strains do not grow

tested concentration range. No obvious inhibition of 5-HMF on *T. cutaneum* 2.1374 was observed in the experimental range. The cell growth and lipid production on the other five strains decreased with increasing 5-HMF concentration, but the deduction was not as significant as that by furfural. Only *L. starkeyi* 2.1608 and 2.1390 ceased to growth in the experimental range of 5-HMF.

Screening of the Oleaginous Yeasts Under the Inhibition by Phenol Derivatives from Lignin

Lignin is a complicated polymer of phenol compounds, composed of three monomers of p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol through the alkyl–aryl, alkyl–alkyl, and aryl–aryl ether bonds. Lignin could be converted into various phenol derivatives in the pretreatment processing, especially in the chemical-based pretreatment such as dilute acid pretreatment. Phenol compounds from lignin degradation had been long recognized as the inhibitors for ethanol fermentation, but its inhibitory performance on oleaginous yeast was not yet well characterized. In this work, the typical lignin degradation compounds, vanillin and hydroxybenzaldehyde, were selected as the model inhibitors to investigate the inhibition effect of lignin degradation products on oleaginous yeasts.

Table 3 shows the results of the effect of vanillin to minimum nutritious medium. For vanillin, the minimum amount of it can significantly inhibit the cell growth and lipid production with the only exception of *T. cutaneum* 2.1374. *L. starkeyi* 2.1608, *R. toruloides* 2.1389, and *R. glutinis* 2.107 ceased to grow under the very low concentration of vanillin (less than 1 g/L) or even died when the smallest dosage of vanillin was added in the experimental range (0.5 g/L). *R. glutinis* 2.704 performed a better vanillin tolerance and ceased to grow when vanillin was greater than 2 g/L. No obvious inhibition of vanillin on *T. cutaneum* 2.1374 in the experimental range. Oppositely the cell growth and lipid production

of *T. cutaneum* 2.1374 reached the maximum when vanillin was added to 0.5 g/L, in which the DCM and lipid were 3.41 and 1.17 g/L, respectively.

For hydroxybenzaldehyde, Table 3 showed that the cell growth and lipid production of the selected six strains decreased with the increasing concentration of hydroxybenzaldehyde in the experimental range. However the inhibition of hydroxybenzaldehyde was not as strong as that of vanillin. Again, *T. cutaneum* 2.1374 showed the best tolerance and resistance to hydroxybenzaldehyde, and the only strain among the selected six to grow in the maximum concentration of hydroxybenzaldehyde, although its growth decreased with the increasing hydroxybenzaldehyde concentration. The other strains ceased to grow at the 1.5 g/L of hydroxybenzaldehyde.

The cumulative inhibition on *T. cutaneum* 2.1374 by the major inhibitors, acetic acid, furfural, and 5-HMF, was added and the result was shown in Fig. 3. The major compositions in medium include glucose 28.18 g/L, xylose 19.08 g/L, acetic acid 1.24 g/L, levulinic acid 2.66 g/L, furfural 0.25 g/L, 5-HMF 0.71 g/L. Figure 3 showed that under the cumulative inhibition of acetic acid, furfural, and 5-HMF, the cell growth of *T. cutaneum* 2.137, glucose or xylose consumption, and inhibitor consumption was similar to that under the inhibition of individual inhibitors.

Cultivation of *T. cutaneum* 2.1374 in Corn Stover Hydrolysate

The corn stover hydrolysate was obtained using the method in the "Materials and Methods" section. The inhibition effect of the combinational degradation products from lignocellulose on *T. cutaneum* 2.1374 was carried out in the corn stover hydrolysate as the culture medium. The hydrolysate was diluted by the synthetic medium to 80%, 60%, 40%, and 20% (hydrolysate content). The time courses of the cell growth and the consumption of glucose, xylose, and acetic acid were shown in Fig. 4.

The obvious lag phase was observed in the fermentation profile of each dilution medium. The lag time increased with the increasing content of hydrolysate: the fermentation started with almost no lag time when the hydrolysate was diluted to 20% and 40%; lag time was 24, for the media with 60%. The fermentation did not happen for the media with 80% dilutions, respectively. Only 20% had xylose consumption. Figure 4b showed an interesting phenomenon for acetic acid metabolism. Obviously acetic acid

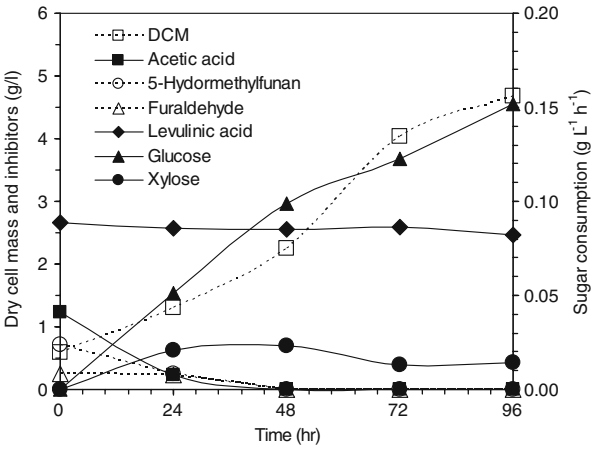

Fig. 3 Time course of the *T. cutaneum* 2.1374 fermentation under the cumulative effect of the major inhibitors. The major compositions in medium include glucose 28.18 g/L, xylose 19.08 g/L, acetic acid 1.24 g/L, levulinic acid 2.66 g/L, furfural 0.25 g/L, 5-HMF 0.71 g/L. All cultures were incubated in an orbital shaker at an agitation rate of 180 rpm and incubation temperature 30 °C

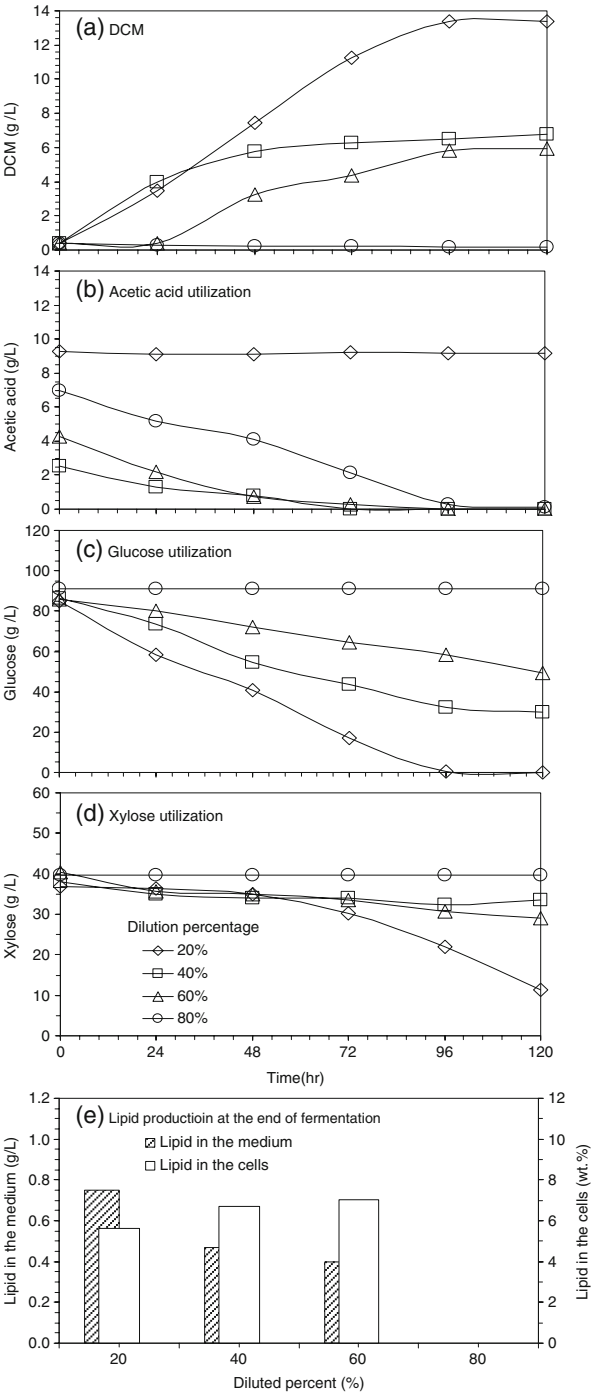

Fig. 4 Time course of the *T. cutaneum* 2.1374 in lignocellulosic hydrolysate fermentation. *Symbols* indicate the different dilutioin ratio lignocellulosic hydrolysate with the nitrogen-limited medium whose sugar and nitrogen composition were same with hydrolysate. **a**–**d** The concentration change of DCM, acetic acid, glucose, xylose verse fermentation time, respectively. **e** Lipid production at the end of fermentation. *Symbols* indicate the different dilution ratio lignocellulosic hydrolysate with the nitrogen-limited medium whose sugar and nitrogen composition were the same with hydrolysate

participated the metabolism of *T. cutaneum* 2.1374 for production of lipid and seemed that there was correlation between the fermentation lag time and the acetic acid. In other words, the fermentation and lipid production started when the residual acetic acid was consumed to below a certain value (about 2–3 g/L). The result was in agreement with that acetic acid as the sole inhibition ("Screening of the Oleaginous Yeasts under the Inhibition of Organic Acids"). Figure 4e showed that lipid in medium and lipid in cell were much lower than the fermentation in minimum nutritious medium. And with the increasing of hydrolysate content, the inhibition was much stronger. When the hydrolysate content was to 80%, the *T. cutaneum* 2.1374 creased to grow completely.

Discussion

In this paper, the most adopted oleaginous strains were screened based on their inhibitory performance of the lignocellulose degradation compounds. The tolerance and stress of the degradation inhibitors on the sugar utilization of lignocellulose-derived glucose and xylose, the cell growth, and lipid accumulation properties were quantitatively studied. Based on the results, an optimal strain *T. cutaneum* 2.1374 tolerant to the degradation products was screened for the use of lignocellulose hydrolysate fermentation. Seven typical lignocellulose degradation products formed in various pretreatment and hydrolysis processing were selected as the model inhibitors, including three organic acids: acetic acid, formic acid, and levulinic acid; two furan compounds: furfural and 5-HMF; two phenol derivatives: vanillin and hydroxybenzaldehyde. These compounds represent the major degradation products from lignocellulose. The effect of these compounds on the ethanol fermentation strains were already well characterized, but the effect on oleaginous yeast strains remained almost untouched.

The minimum nutritious medium under the nitrogen-limiting condition were applied to carry out all the experiments without adding any expensive complex ingredients such as yeast extract, peptone, etc. The addition of these expensive ingredients certainly improved the fermentation performance and lipid productivity, but the high cost made the lipid produced too expensive to be used as biodiesel feedstock. We clearly and consistently keep on using the minimum nutritious medium in which glucose and xylose were the sole carbon source, ammonia sulfate was the sole nitrogen source, and two inorganic salts (KH_2PO_4, $MgSO_4$). The selected oleaginous yeast strains were prescreened using the minimum nutritious medium under the nitrogen limiting conditions. Four of the ten oleaginous strains were deleted because of their poor performance on the cell growth and lipid production in the minimum nutritious medium. The rest of the six strains were selected for the further inhibitory effect experiment, including *T. cutaneum* 2.1374, *R. toruloides* 2.1389, *L. starkeyi* 2.1390, *L. starkeyi* 2.1608, *R.glutinis* 2.704, and *R. glutinis* 2.107.

For the inhibitory effect of organic acids, acetic acid was found to be the strongest inhibitors among the three selected organic acids. *T. cutaneum* 2.1374 was the only survival among the six candidates under 5 g/L of acetic acid. It should be noticed that acetic acid is the inevitable compound from the hemicellulose hydrolysis, and 5 g/L acetic acid is just a low index for the lignocellulose hydrolysate (depending on the different pretreatment, the concentration of acetic acid may reach as high as 10–20 g/L [18]). Formic acid is also a strong inhibitor, and again, *T. cutaneum* 2.1374 showed the best tolerance to it. The inhibition on the selected oleaginous yeasts by levulinic acid was weak compared to acetic acid and formic acid. For *T. cutaneum* 2.1374, no obvious inhibitory effect was observed on its cell growth and lipid production.

For the inhibitory effect of furan compounds, the xylose degradation furfural was found to be a strong inhibitor. Most strains ceased to grow under the minimum presence of furfural with the only exception of *T. cutaneum* 2.1374. On the other hand, 5-HMF, the glucose degradation product and a furan derivative with similar molecular structure to furfural, was found not a strong inhibitor, although the cell growth and lipid production decreased with the increasing 5-HMF concentration. No obvious inhibitory effect for *T. cutaneum* 2.1374 by 5-HMF was observed on its cell growth and lipid production.

For the inhibitory effect of phenol derivatives, vanillin was found to be a strong inhibitor similar to acetic acid and furfural. All strains ceased to grow except *T. cutaneum* 2.1374. Hydroxybenzaldehyde was a weak inhibitor to the selected strains, and again, *T. cutaneum* 2.1374 showed the best tolerance to hydroxybenzaldehyde.

Based on the results of the inhibitory experiment above, *T. cutaneum* 2.1374 always was the most adopted strain for each group of the inhibitors from lignocellulose degradation compounds, either organic acid, furan compounds, or phenol derivatives, among the selected oleaginous yeast strains. The strain was further tested in the real corn stover hydrolysate for production of lipid, and the results showed that the combinational inhibitory by the full spectrum of lignocellulose degradation products in the hydrolysate were strengthened comparing to that of the single inhibition, indicated by the lag time before the lipid fermentation started. It was worth notifying that *T. cutaneum* 2.1374 was able to metabolize acetic acid, formic acid, levulinic acid slowly simultaneously with the glucose or xylose consumption. In the hydrolysate fermentation, the fermentation lag time was found to be closely related to the concentration of acetic acid. Only acetic acid was reduced to a certain value, the fermentation could start and the lipid began to accumulate. However, although acetic acid and other organic acids participated the metabolism, the lipid did not increased correspondingly to the organic acid consumption rate, indicating the metabolic pathway of organic acids for *T. cutaneum* 2.1374 is complicated than that of the sugars.

As an environmental organism usually used for the treatment of waste water, the performance of *T. cutaneum* 2.1374 for the tolerance of lignocellulose degradation products was understandable. The outstanding tolerance to inhibitors, the efficient uptake of the minimum nutritious substances, and the sufficient utilization of glucose and xylose which were the major fermentable sugars from the hydrolysis of lignocellulosic biomass made *T. cutaneum* 2.1374 to be one of the candidates of industrial importance for lipid production using lignocellulose material. The further research on *T. cutaneum* 2.1374 and its mutant strains is going on in our laboratory for production of lipid using lignocellulose hydrolysate as carbon source. A very preliminary estimation is made as following: the current market price of diesel or biodiesel is about $1100/ton. Since the lipid oil cost generally takes 70–80% of the total biodiesel cost, the price for the lipid for production of biodiesel should be around $900/ton. On the other hand, the cost for fermentable sugars from the lignocellulosic feedstock is approximately $180/ton based on our hydrolysis experiment (data not shown). The current yield from sugars to lipid in this study is approximately 10%, such the cost for the lipid is approximately $1,800/ton. That means that at the present fermentation level, the cost of oleaginous yeast lipid is almost doubled than the current economic price of the lipid in biodiesel production. To serve the meaningful biodiesel feedstock, the oleaginous yeast lipid should cut at least 50% of its production cost.

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