



## Converting lignin derived phenolic aldehydes into microbial lipid by *Trichosporon cutaneum*

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### ABSTRACT

Lignin is one of the major components of lignocellulose biomass and chemically degrades into phenolic aldehydes including 4-hydroxybenzaldehyde, vanillin, and syringaldehyde. No lipid accumulation from the phenolic aldehydes by oleaginous microbes had been succeeded. Compared with vanillin and syringaldehyde, *T. cutaneum* ACCC 20271 have better tolerance to 4-hydroxybenzaldehyde. 4-Hydroxybenzaldehyde was found to be able as the substrate for lipid accumulation, while vanillin and syringaldehyde were only converted to less toxic phenolic alcohols and acids without observable lipid accumulation, perhaps due to the space shelling of methoxyl group(s) in the structures. A long term fed batch fermentation of 4-hydroxybenzaldehyde accumulated 0.85 g L<sup>-1</sup> of lipid, equivalent to 0.039 g lipid per gram of 4-hydroxybenzaldehyde substrate, approximately 3.7 folds greater than the control without 4-hydroxybenzaldehyde addition. The fatty acid composition well met the need for biodiesel synthesis. The preliminary pathway from 4-hydroxybenzaldehyde to lipid was predicted. This study took the first experimental trial on utilizing phenolic aldehydes as the sole carbon sources for microbial lipid accumulation by *T. cutaneum* ACCC 20271.

### 1. Introduction

Lignin is one of the three major components of lignocellulose biomass. Different from the polysaccharide of cellulose and hemicellulose, lignin is a cross-linked phenylpropanoid polymer composed of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) groups (Chen and Wan, 2017). When lignin is degraded into its monomer compounds chemically or biologically (Zhu et al., 2017), the major degradation products include 4-hydroxybenzaldehyde, vanillin, and syringaldehyde, or their corresponding reduced or oxidized products (alcohols or acids), representing the degradation of H, G, and S groups, respectively. After cellulose and hemicellulose in lignocellulose biomass are utilized in the upstream biorefinery production of fuels and chemicals, the lignin residue is generally used solid fuel for electricity generation through combustion due to its high recalcitrance to bioconversion. The value added utilization of lignin through bioconversion has been proved to be a tough task, except for the potential lipid synthesis. Several studies tried to convert the oxidized lignin monomers into microbial lipid by oleaginous yeasts. However, these studies only concerned the conversion from the less toxic phenolic acid compounds, instead of the original phenolic aldehyde compounds due to the high inhibitory property. Shields-Menard et al. (2017) used phenolic acids (4-hydroxybenzoic acid and vanillic acid) as the carbons sources to

accumulate the microbial lipid up to a maximum of 0.11 g L<sup>-1</sup> approximately by *Rhodococcus rhodochrous*. Kosa and Ragauskas (2012) used 4-hydroxybenzoic acid to accumulate 0.07 g L<sup>-1</sup> of lipid approximately by *Rhodococcus opacus* DSM 1069. However, no trials had been made on utilizing phenolic aldehyde monomers of lignin such as 4-hydroxybenzaldehyde, vanillin and syringaldehyde to microbial lipid due to their high toxicity to general microorganisms and their recalcitrant to bioconversion.

*Trichosporon cutaneum* is an oleaginous yeast with high tolerance to lignocellulosic inhibitors and able to accumulate high microbial lipid using lignocellulosic feedstock (Chen et al., 2009; Gao et al., 2014; Wang et al., 2016a). Our previous study shows that the highly expressed alcohol dehydrogenases, aldehyde reductase and aldehyde dehydrogenase of *T. cutaneum* provide the strong capacity on degrading lignin derived monomers (Wang et al., 2016a, 2016b). In this study, the three major lignin derivatives, 4-hydroxybenzaldehyde, vanillin, and syringaldehyde, or their corresponding reduced or oxidized products (alcohols or acids), representing the degradation production of H, G, and S groups, were selected as the substrates of *T. cutaneum* ACCC 20271 for lipid synthesis. The results showed that 4-hydroxybenzaldehyde was completely converted to lipid by *T. cutaneum*, while vanillin and syringaldehyde were only reduced into less toxic phenolic alcohols and acids without the observable microbial lipid

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accumulation. This study took the first insight into the lipid accumulation from the phenolic aldehydes from lignin degradation by a high inhibitor tolerant yeast *T. cutaneum*. The results and methodology developed in this study provided an important way of lignin utilization for value added high energy content fuel through bioconversion pathway in the future biorefinery industry.

## 2. Materials and methods

### 2.1. Reagents

4-Hydroxybenzaldehyde, vanillin and syringaldehyde were purchased from Sangon Biotech, Shanghai, China. Yeast extract and peptone were from Oxiod, Basingstoke, Hampshire, UK. All other chemicals were of analytical reagent grade and purchased from Lingfeng Chemical Reagent Co., Shanghai, China.

### 2.2. Strains and media

*T. cutaneum* ACCC 20271 was obtained from Agricultural Culture Collection of China (ACCC, <http://www.accc.org.cn>), Beijing, China. The strain was maintained on YPD agar petri dish containing 10 g L<sup>-1</sup> of yeast extract, 20 g L<sup>-1</sup> of peptone, 20 g L<sup>-1</sup> of glucose, and 20 g L<sup>-1</sup> of agar, then transferred into YPD medium for seed culture. The synthetic medium used for lipid fermentation and inhibitor degradation experiment contained 0.5 g L<sup>-1</sup> of yeast extract, 0.22 g L<sup>-1</sup> of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g L<sup>-1</sup> of MgSO<sub>4</sub>·7H<sub>2</sub>O, and 1.0 g L<sup>-1</sup> of KH<sub>2</sub>PO<sub>4</sub> and appropriate 4-hydroxybenzaldehyde, vanillin, or syringaldehyde.

### 2.3. Lipid fermentation

Lipid fermentation was carried out in 500 mL shake flasks containing 50 mL of the synthetic medium. One single colony of *T. cutaneum* ACCC 20271 was transferred into 20 mL of YPD medium for overnight culture, then 10% (v/v) of the culture broth was inoculated into 50 mL of YPD medium and cultured for 24 h as the seed broth. Finally, 10% (v/v) of the seed broth was inoculated into the synthetic media containing lignin monomers and fermented at 30 °C and 180 rpm. Samples were collected periodically to measure the concentration of lignin monomers and their intermediates.

The fed batch fermentation was conducted using 4-hydroxybenzaldehyde as the sole carbon source in the synthetic fermentation medium. Every 24 h 4-hydroxybenzaldehyde was added to the concentration of 1 g L<sup>-1</sup> and cultured for 480 h (20 days). Samples were collected every 48 h (twice addition period of 4-hydroxybenzaldehyde). All experiments were performed for three times and the error ranges were given in the figures.

### 2.4. Lipid extraction

30 mL of the fermentation broth was centrifuged to collect the yeast cells, washed and dried at 80 °C to constant weight, then the dry cell weight (DCW) was measured. The lipid was extracted using chloroform-methanol method (Folch et al., 1957). Briefly, the dried cells were transferred into 6 mL of 4 M HCl solution, boiled for 10 min and then quickly cooled on ice. The slurry was mixed with 20 mL of chloroform-methanol solution (2:1, v/v), and vigorously shaken at 30 °C for 1 h. The lipid containing chloroform was obtained by centrifugation and the lipid was obtained by vacuum evaporation at 80 °C and measured gravitationally.

The extracted lipid was transmethylated before fatty acid composition determination. Briefly, 5 mL of 0.5 M KOH-methanol was added into the lipid for saponification at 60 °C for 1 h, then 4 mL of boron trifluoride-methanol (2:5, v/v) was added for esterification for 30 min. After the mixture was cooled down, 5 mL of hexane and 2 mL of the saturated NaCl solution were separately added and mixed well. After

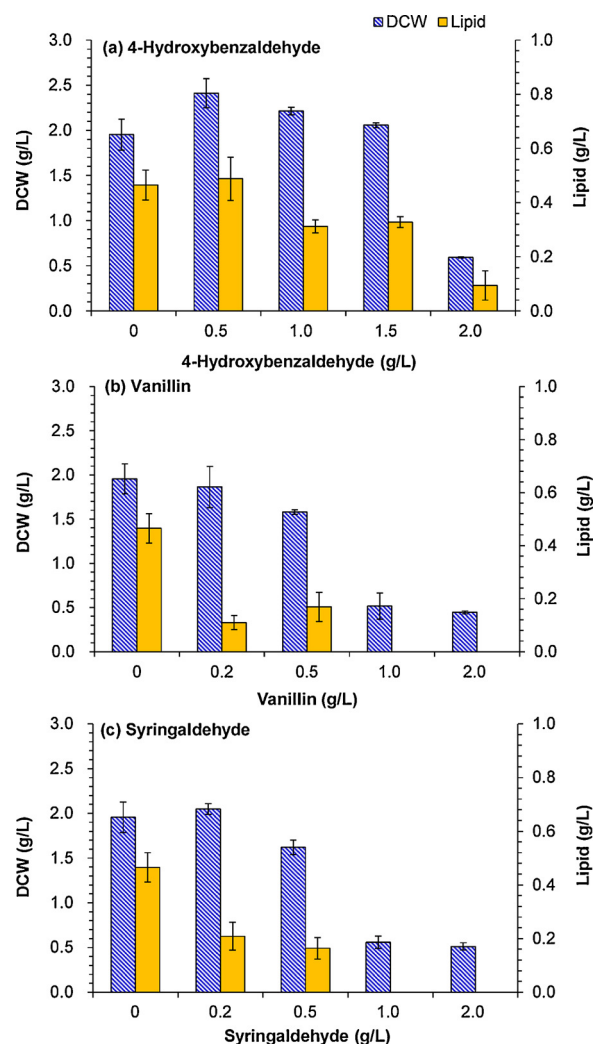


Fig. 1. Cell growth and lipid accumulation in different concentration of phenolic aldehydes by *T. cutaneum* ACCC 20271. (a) 4-Hydroxybenzaldehyde; (b) Vanillin; (c) Syringaldehyde. The culture was carried out at 30 °C, 180 rpm with an inoculum size of 10% (v/v) into 50 mL synthetic medium in a 500 mL flask. All samples were taken after 120 h fermentation.

centrifugation for at 10,000 rpm for 5 min, the upper hexane solution containing fatty acid methyl esters (FAMES) (Morrison and Smith, 1964) was collected for GC–MS analysis to test the fatty acid composition. The GC–MS was operated at the injector temperature 280 °C and 1 mL/min of nitrogen gas with the temperature gradient of 16 °C/min from 80 °C for 3 min till 280 °C then held for 8 min.

### 2.5. Inhibitor analysis

4-Hydroxybenzaldehyde, 4-hydroxybenzoate, 4-hydroxybenzylalcohol, vanillin, vanillic acid, vanillyl alcohol, syringaldehyde, syringate and syringic alcohol were analyzed using HPLC (UV/Vis detector SPD-20 A, Shimadzu, Kyoto, Japan) fitted with YMC-Pack ODS-A column (YMC, Kyoto, Japan) at 35 °C. 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 a 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 (Khoddami et al., 2013).

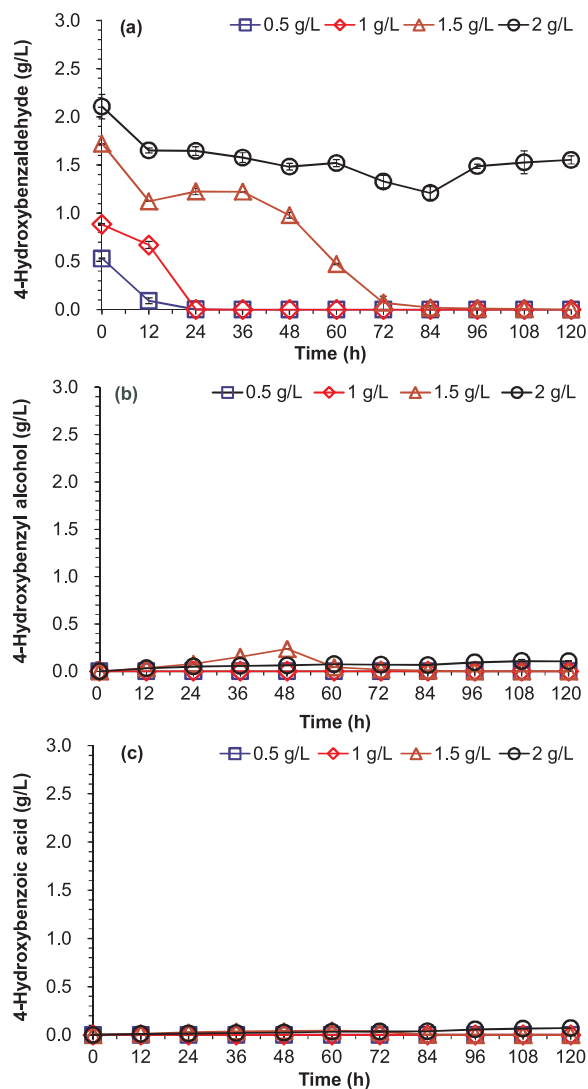


Fig. 2. Biodegradation of 4-hydroxybenzaldehyde (a) and its alcohol (b) and acid (c) derivatives by *T. cutaneum* ACCC 20271. The culture was carried out at 30 °C, 180 rpm with an inoculum size of 10% (v/v) into 50 mL synthetic medium in a 500 mL flask.

### 3. Results and discussion

#### 3.1. Phenolic aldehyde tolerance and conversion of *T. cutaneum* ACCC 20271

Three phenolic aldehydes, 4-hydroxybenzaldehyde, vanillin, and syringaldehyde, were selected to represent the typical lignin degradation monomers of hydroxyphenyl group (H), guaiacyl group (G), and syringyl group (S). To examine the phenolic aldehyde conversion by microbial cells, the tolerance is the first and the most important consideration to validate the feasibility of the bioconversion. This study examined the cell growth and lipid accumulation of *T. cutaneum* ACCC 20271 under the stress of the three phenolic aldehydes (Fig. 1). *T. cutaneum* ACCC 20271 demonstrated the significant different tolerance to these representative inhibitors. 4-Hydroxybenzaldehyde at 1.0–1.5 g L<sup>-1</sup> showed the negligible inhibition on cell growth and lipid accumulation, even showed observable cell growth and lipid accumulation at the highest 2.0 g L<sup>-1</sup> of 4-hydroxybenzaldehyde (Fig. 1a). On the other hand, 0.1 g L<sup>-1</sup> of vanillin significantly inhibited the cell growth and 0.2 g L<sup>-1</sup> of vanillin reduced its lipid accumulation to 20% of the control (Fig. 1b). Syringaldehyde showed the very similar inhibition on

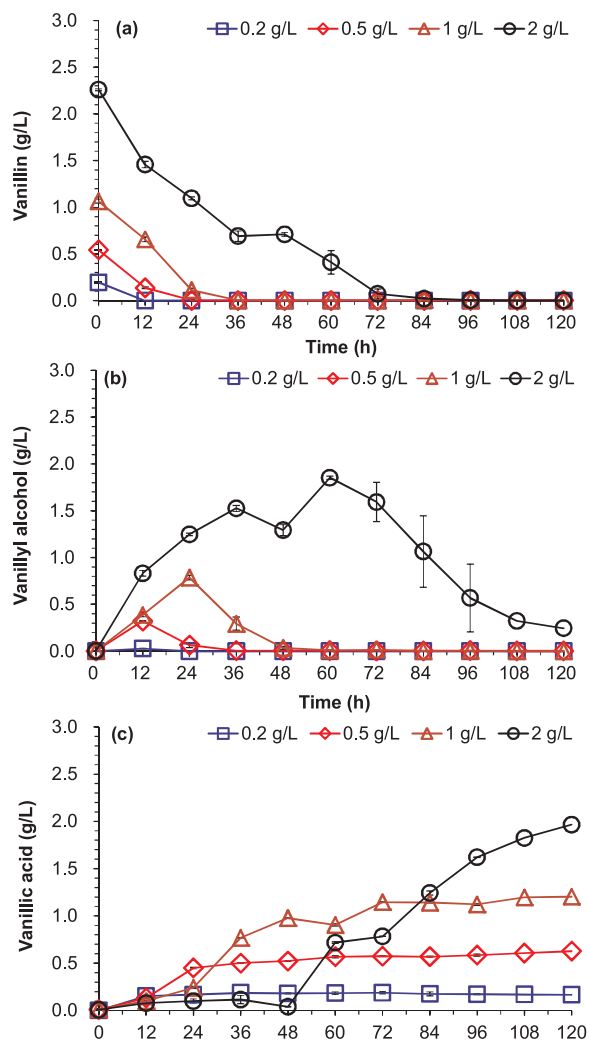
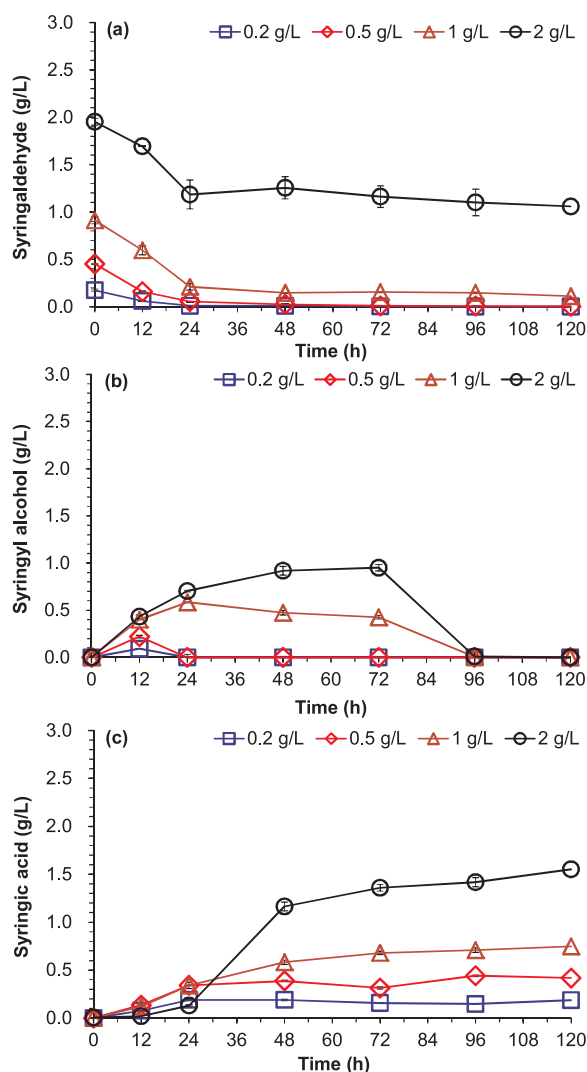


Fig. 3. Biodegradation of vanillin (a) and its alcohol (b) and acid (c) derivatives by *T. cutaneum* ACCC 20271. Culture conditions: The culture was carried out at 30 °C, 180 rpm with an inoculum size of 10% (v/v) into 50 mL synthetic medium in a 500 mL flask.

*T. cutaneum* ACCC 20271. (Fig. 1c).

The microbial tolerance is generally regarded as its capacity to convert the toxic inhibitors into less toxic metabolites (Liu et al., 2009; Liu and Moon, 2009). The conversion performance of the phenolic aldehydes by *T. cutaneum* ACCC 20271 were then examined in (Figs. 2–4). Corresponding to the differences in phenolic aldehyde tolerance, 4-hydroxybenzaldehyde showed the quick and complete conversion by *T. cutaneum* within 24 h (Fig. 2). No accumulation of the corresponding metabolites 4-hydroxybenzyl alcohol or 4-hydroxybenzoic acid at the initial 4-hydroxybenzaldehyde concentrations of 0.5 and 1.0 g L<sup>-1</sup>. When 4-hydroxybenzaldehyde increased to 1.5 g L<sup>-1</sup>, 4-hydroxybenzyl alcohol and 4-hydroxybenzoic acid started the accumulation but eventually was completely degraded after 84 h without the observable accumulation. When 4-hydroxybenzaldehyde increased to 2 g L<sup>-1</sup>, the conversion of 4-hydroxybenzaldehyde was not complete and the slight accumulation of 4-hydroxybenzyl alcohol and 4-hydroxybenzoic acid maintained at low level after 120 h culture, indicating the 2 g L<sup>-1</sup> of 4-hydroxybenzaldehyde exceeded the upper limit of tolerance of *T. cutaneum* ACCC 20271. Fig. 2(b) and (c) indicate that the alcohol and acid from the 4-hydroxybenzaldehyde conversion were very low, but the residual alcohol and acid concentrations increased slightly with the increasing 4-hydroxybenzaldehyde concentration, indicating the conversion capacity of *T. cutaneum* is already



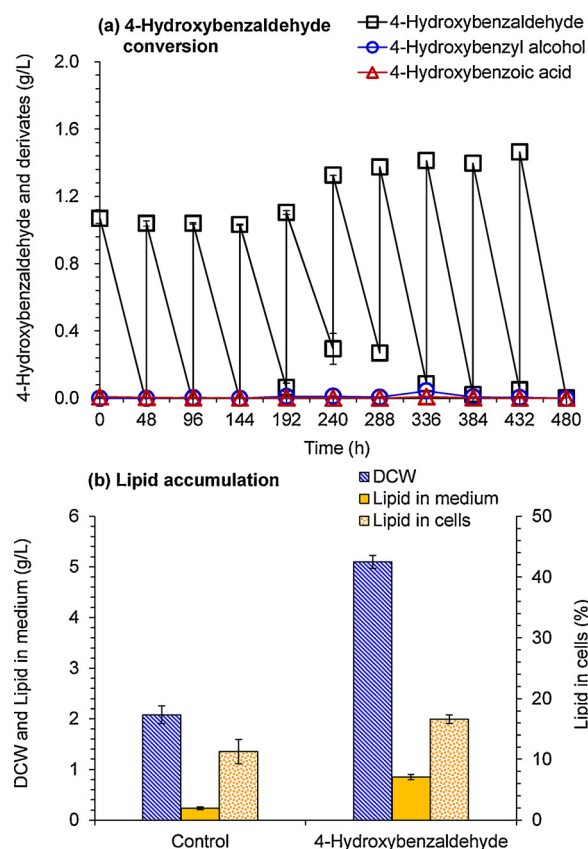
**Fig. 4.** Biodegradation of syringaldehyde (a) and its alcohol (b) and acid (c) derivatives by *T. cutaneum* ACCC 20271. The culture was carried out at 30 °C, 180 rpm with an inoculum size of 10% (v/v) into 50 mL synthetic medium in a 500 mL flask.

at the edge of full conversion and the further increase of 4-hydroxybenzaldehyde might lead to the incomplete conversion with the residual alcohol and acid existences. The conversion capacity of vanillin and syringaldehyde by *T. cutaneum* ACCC 20271 were similar in the formation of the intermediates (Figs. 3 and 4). Different from the fast and complete conversion of 4-hydroxybenzaldehyde (below its maximum tolerable concentration  $2.0 \text{ g L}^{-1}$ ), vanillin and syringaldehyde were quickly converted into the corresponding alcohols (vanillyl alcohol and syringyl alcohol), then the acids (vanillic acid and syringic acid), respectively, but these phenolic acids failed to be ultimately degraded and finally the acids accumulated.

These results suggest that 4-hydroxybenzaldehyde behaved the potential of lipid formation by *T. cutaneum* ACCC 20271 because of its complete conversion capacity as a carbon source, while vanillin and syringaldehyde were less possible to be used as carbon source for lipid accumulation in the range of experiment.

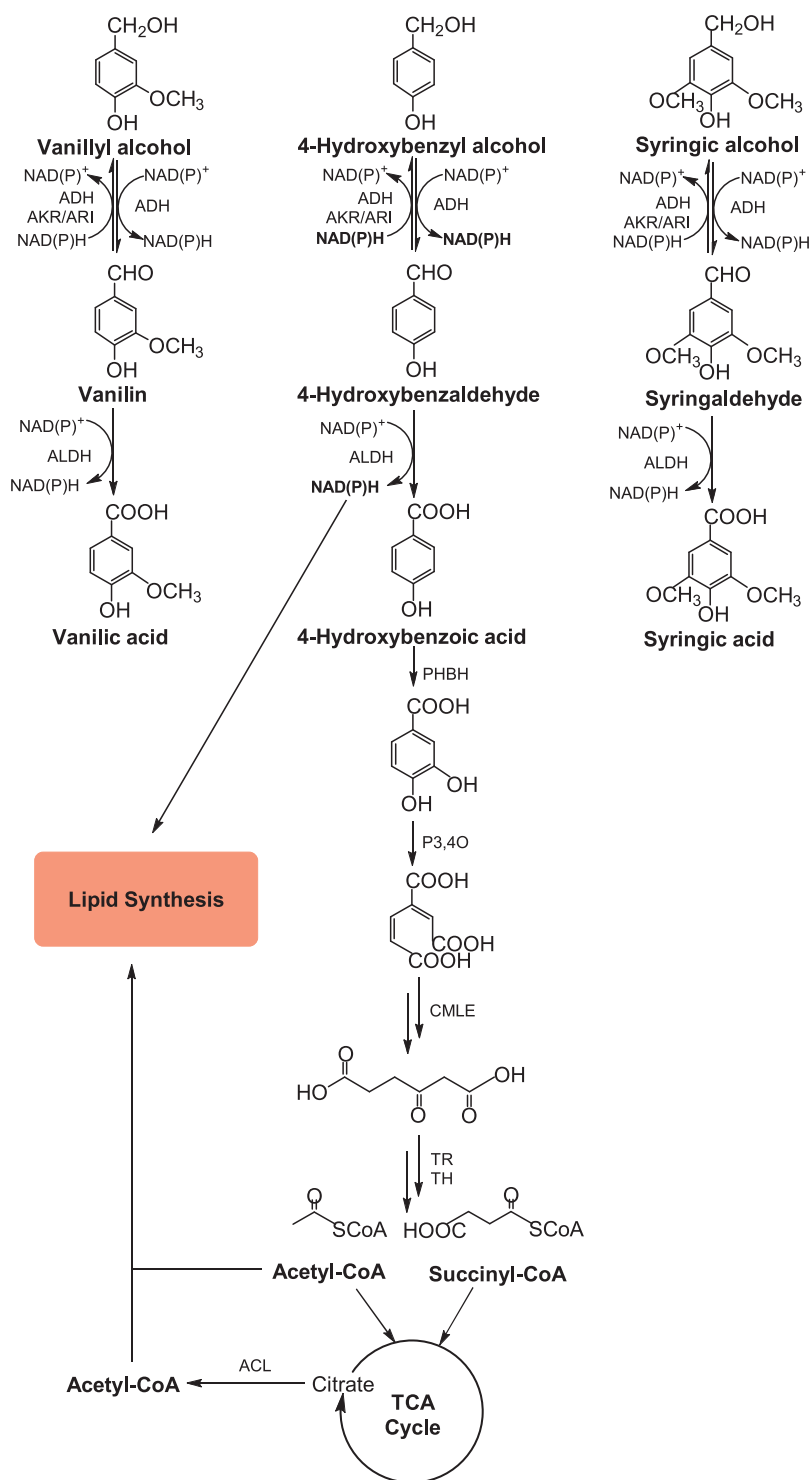
### 3.2. Lipid accumulation from 4-hydroxybenzaldehyde by *T. cutaneum* ACCC 20271

To verify the lipid accumulation potential using 4-hydroxybenzaldehyde as the sole carbon source, a long term fed batch



**Fig. 5.** Long term fed batch fermentation of 4-hydroxybenzaldehyde as the sole carbon source for lipid fermentation by *T. cutaneum* ACCC 20271. (a) Time courses of 4-hydroxybenzaldehyde and its derivatives; (b) Lipid accumulation in *T. cutaneum* ACCC 20271 cells. Control is the synthetic fermentation medium without any 4-hydroxybenzaldehyde addition. The fermentation was carried out at 30 °C, 180 rpm with an inoculum size of 10% (v/v) into 50 mL synthetic medium in 500 mL flasks.

fermentation of *T. cutaneum* ACCC20271 was conducted by the periodical addition of 4-hydroxybenzaldehyde to the mild concentration of  $1 \text{ g L}^{-1}$  every 24 h (Fig. 5). The result showed that 4-hydroxybenzaldehyde was completely consumed in each fed batch of 4-hydroxybenzaldehyde without accumulation of 4-hydroxybenzyl alcohol and 4-hydroxybenzoic acid, indicating a complete conversion of 4-hydroxybenzaldehyde into either cell growth or metabolites formation (Fig. 5a). Starting from 240 h, the conversion rate of 4-hydroxybenzaldehyde declined and its residual concentration increased perhaps due to the harsh stress of inhibitor property of 4-hydroxybenzaldehyde on the cell growth and metabolism of *T. cutaneum*. However, the residual 4-hydroxybenzaldehyde decreased in the consequent culture from 288 h, perhaps due to the effect of adaptive evolution of *T. cutaneum* to the culture environment. After the 480 h fed batch culture, the final cell mass of the fed batch addition of 4-hydroxybenzaldehyde was  $5.1 \text{ g L}^{-1}$  approximately 145% greater than the control (without 4-hydroxybenzaldehyde addition); the lipid content in cells was 16.6%, approximately 48% greater than then control; the lipid titer was  $0.85 \text{ g L}^{-1}$ , approximately 265% greater than the control (Fig. 5b). This result was also eight folds greater than that the best lipid accumulation from 4-hydroxybenzoic acid (the metabolite of 4-hydroxybenzaldehyde) as the substrate ( $0.11 \text{ g L}^{-1}$ ) by *Rhodococcus rhodochrous* (Shields-Menard et al., 2017). The fatty acid composition of the lipid was measured to be 42.9% of stearic acid (C18:0), 36.6% of oleic acid (18:1) and 20.5% of palmitic acid (C16:0), similar with that of soybean oil and other vegetable oils (Ramis et al., 2009) used for biodiesel production.



**Fig. 6.** Metabolic pathway for 4-hydroxybenzaldehyde into lipid by *T. cutaneum* ACCC 20271. ADH, Alcohol dehydrogenase; ALDH, Aldehyde dehydrogenase; AKR, Aldo-keto reductase; ARI, Aldehyde reductase; PHBH, para-hydroxybenzoate hydroxylase; P3,O4, protocatechuate 3,4-dioxygenase; CMLE,  $\beta$ -carbon-cis-cis muconate lactonizing enzyme; TR,  $\beta$ -ketoacid/succinyl-CoA transferase; TH,  $\beta$ -ketoacidyl-CoA thiolase; ACL, ATP citrate-lyase.

The putative lipid accumulation pathway (Fig. 6) of *T. cutaneum* ACCC 20271 from 4-hydroxybenzaldehyde was predicted based on the genome annotation of *T. cutaneum* ACCC 20271 and the previous results (Wang et al., 2016a, 2016b; Kosa and Ragauskas, 2012). 4-Hydroxybenzaldehyde is reduced into less toxic 4-hydroxybenzyl alcohol by alcohol dehydrogenases and aldehyde-ketone reductases/aldehyde reductases (with one NAD(P)H consumption). The 4-hydroxybenzyl alcohol is oxidized back to low titer aldehyde (with one NAD(P)H

generation), then further oxidized into 4-hydroxybenzoate by aldehyde dehydrogenases (with one extra NAD(P)H generation). The alcohol oxidase and aldehyde oxidase also played important roles in the oxidation process (Wang et al., 2016a). Vanillin and syringaldehyde follow the similar pathway and are finally converted to their acid forms.

However, the acids are neither able to be further converted into the intermediates to enter the TCA cycle, nor to convert into the precursor compounds for lipid synthesis. The possible reason for incomplete

conversion of vanillin and syringaldehyde by *T. cutaneum* ACCC 20271 might be the existence of methoxyl group(s) in vanillyl acid (one methoxyl group) and syringate (two methoxyl groups) (Palmqvist et al., 1999). The space shelling effect blocked one or more catalytic reactions before finally assimilated into TCA circle. Modification by evolutionary adaption or metabolic engineering on *T. cutaneum* ACCC 20271 might improve the assimilation of vanillyl acid and syringate to the microbial lipid accumulation using the complete lignin monomers.

4-Hydroxybenzoate is further assimilated into protocatechuate by dioxygenases under oxygen as the substrate. In the coming step, protocatechuate is converted into succinyl-CoA and acetyl-CoA via the  $\beta$ -ketoacid pathway (Wells and Ragauskas, 2012). The acetyl-CoA is either directly acts the substrate for lipid synthesis, or assimilated into the TCA cycle and is ultimately implemented as the major precursor towards lipid synthesis.

#### 4. Conclusions

This study experimentally investigated the lipid accumulation of lignin derived phenolic aldehyde by *T. cutaneum* ACCC 20271. The tolerance and biodegradation of *T. cutaneum* ACCC 20271 on the three lignin monomers was experimentally and systematically investigated. 4-hydroxybenzaldehyde at  $2.0 \text{ g L}^{-1}$  while vanillin and syringaldehyde at  $0.2 \text{ g L}^{-1}$  significantly inhibited the lipid accumulation. A long term fed batch fermentation of 4-hydroxybenzaldehyde as the sole carbon by *T. cutaneum* was conducted and  $0.85 \text{ g L}^{-1}$  of lipid was accumulated, approximately 2.4 folds of cell mass and 3.7 folds of lipid accumulation were obtained comparing to the no 4-hydroxybenzaldehyde addition control and the fatty acid composition met the need for biodiesel production.

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