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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^{-1} 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^{-1} approximately by *Rhodococcus rhodochrous*. Kosa and Ragauskas (2012) used 4-hydroxybenzoic acid to accumulate 0.07 g L^{-1} 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 ass 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^{-1} of yeast extract, 20 g L^{-1} of peptone, 20 g L^{-1} of glucose, and 20 g L^{-1} 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^{-1} of yeast extract, 0.22 g L^{-1} of $(NH_4)_2SO_4$, 0.5 g L^{-1} of $MgSO_4{\cdot}7H_2O$, and 1.0 g L^{-1} of KH_2PO_4 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^{-1} 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 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.

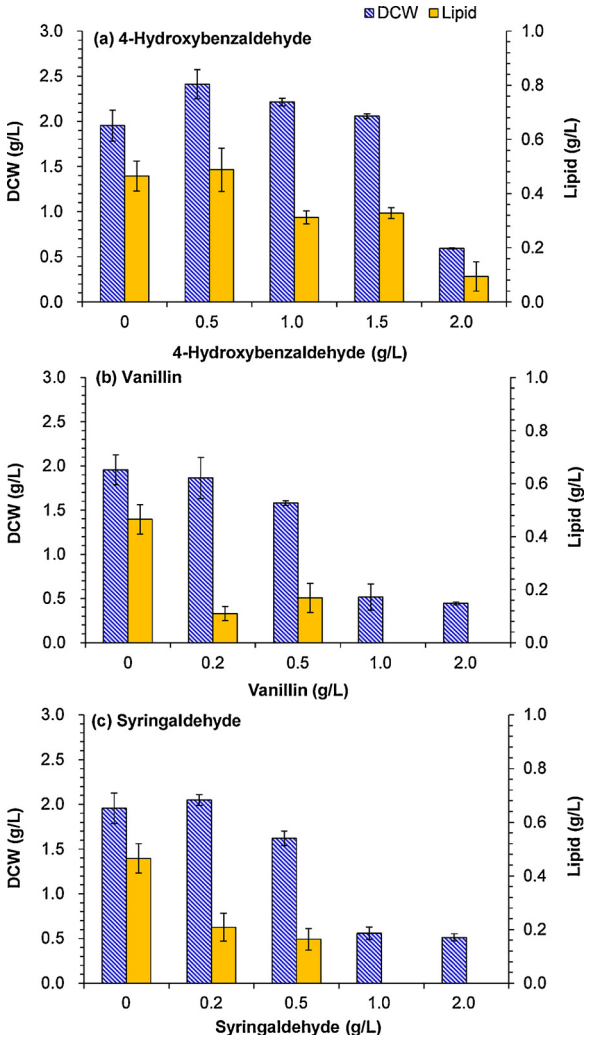

Fig. 1. Cell growth and lipid accumulation in different concentration of phenolic aldehydes by *T. cutaneum* ACCC 20271. (a) 4-Hydroxybenzaldehyde; (b) Vanillin; (c) Syringaldehyde. The culture was carried out at 30 °C, 180 rpm with an inoculum size of 10% (v/v) into 50 mL synthetic medium in a 500 mL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

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).