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Cultivation of lipid-producing bacteria with lignocellulosic biomass: Effects of inhibitory compounds of lignocellulosic hydrolysates

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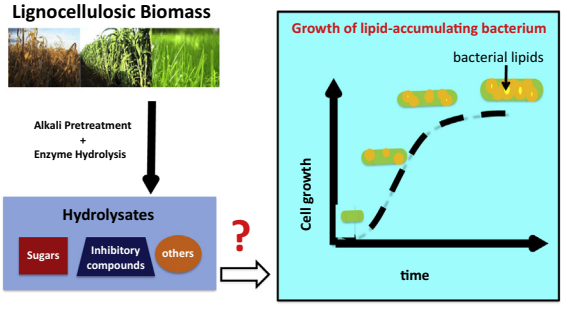
HIGHLIGHTS

- Strain PD630 can tolerate low concentration of five model inhibitory compounds.
- Strain PD630 can use vanillic acid and TPCA for cell growth and TAG accumulation.
- Vanillin can be used as a carbon source, but TAGs are not accumulated.
- Strain PD630 can grow on three lignocellulosic hydrolysates and accumulate TAGs.

GRAPHICAL ABSTRACT



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ABSTRACT

Lignocellulosic biomass has been recognized as a promising feedstock for the fermentative production of biofuel. However, the pretreatment of lignocellulose generates a number of by-products, such as furfural, 5-hydroxylmethyl furfural (5-HMF), vanillin, vanillic acids and *trans-p*-coumaric acid (TPCA), which are known to inhibit microbial growth. This research explores the ability of *Rhodococcus opacus* PD630 to use lignocellulosic biomass for production of triacylglycerols (TAGs), a common lipid raw material for biodiesel production. This study reports that *R. opacus* PD630 can grow well in R2A broth in the presence of these model inhibitory compounds while accumulating TAGs. Furthermore, strain PD630 can use TPCA, vanillic acid, and vanillin as carbon sources, but can only use TPCA and vanillic acid for TAG accumulation. Strain PD630 can also grow rapidly on the hydrolysates of corn stover, sorghum, and grass to accumulate TAGs, suggesting that strain PD630 is well-suited for bacterial lipid production from lignocellulosic biomass.

1. Introduction

Biodiesel is a promising liquid fuel alternative because it is renewable, nontoxic, and contributes much less greenhouse gas emissions to the environment. Biodiesel is made from biolipids, such as triacylglycerols (TAGs) with methanol through a transesterification reaction. Vegetable oils, animal oils/fats, and microbial lipids are available feedstocks for TAGs. However, current biodiesel production is not cost-effective due to the high cost of the feedstocks, which accounts for 70–75% of the total cost of biodiesel production (Chen et al., 2009b). Therefore, it is necessary to exploit a cheaper and more sustainable means for TAG production.

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Lignocellulosic biomass, including forest and agricultural residues and commercial energy crops, represents the most abundant natural resource for the production of advanced biofuels. Fermentation of lignocellulosic biomass to biogas or ethanol has been well developed (Chandra et al., 2012; Mabee et al., 2011; Palmqvist and Hahn-Hagerdal, 2000; Schmitt et al., 2012; Spatari et al., 2010); however, few studies have explored the possibility of producing TAGs from lignocellulosic biomass for biodiesel production (Chen et al., 2009b; Kosa and Ragauskas, 2012; Zeng et al., 2013).

Lignocellulose consists of three types of polymers: 35–55% cellulose, 20–40% hemicellulose, and 10–25% lignin, all of which are strongly intermeshed and chemically bonded (Limayem and Ricke, 2012). Cellulose (consisting of D-glucose only), hemicellulose (consisting of pentoses (xylose and arabinose)) and hexoses (containing mannose, glucose, galactose, etc.) (Galbe and Zacchi, 2012) are bioconvertible. Pretreatment of lignocellulosic biomass is necessary for effective utilization of these carbon sources by biofuel-producing microorganisms (Chiaramonti et al., 2012; Galbe and Zacchi, 2012; Hendriks and Zeeman, 2009; Kwon et al., 2011; Park and Kim, 2012). However, current pretreatment strategies including thermal (hot water, steam) and chemical pretreatments (alkalis, acids or organic solvents) produce unwanted by-products (Chen et al., 2009b; Chiaramonti et al., 2012; Du et al., 2010; Kwon et al., 2011; Nlewem and Thrash Jr., 2010; Park and Kim, 2012). The profile of the pretreatment by-products may vary, depending on the pH and temperature used during the pretreatment. Two common groups of pretreatment by-products are furan and phenol derivatives. The most common furan derivatives are furfural from xylose and 5-hydroxymethylfurfural (5-HMF) from glucose (Almeida et al., 2009; Palmqvist and Hahn-Hagerdal, 2000). Phenol derivatives like vanillin, vanillic acids, and *trans-p*-coumaric acid (TPCA) are lignin degradation products (Du et al., 2010; Kosa and Ragauskas, 2012). These pretreatment by-products released to the downstream process can decrease the efficiency of biofuel production since they are known inhibitors for the growth of microorganisms (Almeida et al., 2009; Du et al., 2010; Kwon et al., 2011).

Bacteria with the ability to overcome the inhibition of these by-products and also use the downstream carbohydrates for producing TAGs would be candidates for bioconverting lignocellulosic biomass into valuable biodiesel. In recent years, several *Rhodococcus* species were demonstrated to have the ability to accumulate TAGs (Alvarez et al., 2000; Kosa and Ragauskas, 2012; Waltermann et al., 2000; Xiong et al., 2012). *Rhodococcus* strains, residing in soil and water environments, are known for their ability to degrade a wide range of compounds (Kosa and Ragauskas, 2012). Several studies have demonstrated that *R. opacus* strain PD630 is capable of accumulating TAGs up to 76% of cell dry weight under nitrogen-deficient conditions (Alvarez et al., 1996, 2000; Waltermann et al., 2000). Accordingly, *R. opacus* PD630 has become one of the most extensively studied TAG-producing microorganism among oleaginous bacterial strains (Alvarez et al., 1996, 2000; Hernandez and Alvarez, 2010; Kosa and Ragauskas, 2012; Waltermann et al., 2000), microalgae (Chen et al., 2009a), yeast (Chen et al., 2009b) and fungi (Zeng et al., 2013) species.

Lignin is known as the most recalcitrant component of lignocellulosic biomass, except through fungal biopulping (Kosa and Ragauskas, 2012). Recent studies have reported that several *Rhodococcus* strains are potential lignin degraders. For example, *R. jostii* RHA1 can breakdown lignocellulose to phenolic products (Ahmad et al., 2010) and *R. opacus* DSM 1069 and PD630 can convert lignin model compounds such as 4-hydroxybenzoic and vanillic acid to produce TAGs (Kosa and Ragauskas, 2012). Furthermore, the successful engineering of xylose (Xiong et al., 2012) and cellobiose (Hetzler and Steinbuchei, 2013; Schmitt et al., 2012) catabolic pathway in *Rhodococcus* strains indicates the possibility that lignocellulosic biomass can be completely utilized by *Rhodococcus* strains to produce TAGs.

In this study, *R. opacus* PD630 was examined for its ability to grow in the presence of model inhibitory compounds of lignocellulosic hydrolysates and to use the model compounds as carbon sources and accumulate TAGs. To better understand the effects of these model inhibitory compounds on cell growth and TAG accumulation, the degradation mechanisms of each model inhibitory compound were studied. Furthermore, the chemical compositions of three hydrolysates from lignocellulosic biomass-corn stover, sorghum, and grass-were determined. The hydrolysates were produced by pretreating the lignocellulosic biomass with sodium hydroxide at low temperature and followed by enzymatic hydrolysis. Alkali pretreatment at low temperature was used in this study because the process has the advantages of effective delignification and retains the hemicellulose in the solids to increase yield (Park and Kim, 2012; Sills and Gossett, 2011). The results of growth tests incorporating these variables were discussed in terms of the suitability of strain PD630 for lipid production from lignocellulosic biomass.

2. Methods

2.1. Materials

Furfural, amido black, and acetic acid were purchased from Fisher Scientific (Pittsburgh, PA). 5-Hydroxymethylfurfural (5-HMF), vanillin, ethyl acetate and hexane were purchased from Acros Organics (Morris Plains, NJ). Vanillic acid, *trans-p*-coumaric acid (TPCA) and cellulase were purchased from Tokyo Chemical Industry Co., LTD (Japan). Glyceryl trioleate (TL), N, O-Bis (trimethylsilyl) trifluoro-acetamide (BSTFA) with trimethylchlorosilane (TMCS), pridine, xylose, glucose, arabinose, *o*-methoxylamine HCl, acetic anhydride were obtained from Sigma–Aldrich (St. Louis, MO). Glucosidase was purchased from MP Biomedicals (Solon, OH). [1,2- $^{13}C_2$] D-glucose was purchased from Cambridge Isotope Laboratories, Inc. (MA, USA). *Rhodococcus opacus* PD630 (DSM 44193, hereafter referred as strain PD630) was purchased from DSMZ, Germany. The strain was streaked on Reasoner's 2A (R2A) agar plates for short-term (2–3 weeks) preservation.

2.2. Effects of inhibitory compounds on cell growth and TAG production

To determine the maximum concentrations of inhibitory compounds that strain PD630 can tolerate, inhibition screening tests were conducted on R2A agar plates containing each of the model inhibitory compounds at different concentrations. For furfural and 5-HMF, the concentrations ranging from 0.05 to 5 g/L were used. For vanillin, vanillic acid and TPCA, the concentrations ranging from 0.05 to 2 g/L were used. The selection of concentration range for each inhibitory compound was based on reported values in different lignocellulosic hydrolysates (Almeida et al., 2009; Chen et al., 2009b). All plates were incubated at 30 °C for 5 days and checked daily for colony formation.

Results of the inhibitory screening tests were used to guide the experimental design of liquid growth tests to determine the inhibitory effects on bacterial cell growth and TAG accumulation. The experiments were conducted in 250-ml flasks containing 100 ml R2A broth amended with 0.2 g/L of one of the inhibitory compounds. The growth media were shaken for 1 h at 37 °C at 200 rpm to ensure complete dissolution of the inhibitory compounds and then filter-sterilized using a 0.22 μm Millex-GP syringe filter unit. A cell suspension pregrown from a single colony in 5 ml of R2A broth for 24–36 h at 150 rpm at 30 °C was used to inoculate the flasks. If not specified, a dilution ratio of 1:30 was applied to reach an initial optical density at 600 nm (OD_{600}) of ~0.1 in each flask. The flasks were then incubated at 30 °C at 150 rpm and