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Consolidated bioprocessing of untreated switchgrass to hydrogen by the extreme thermophile *Caldicellulosiruptor saccharolyticus* DSM 8903 ☆

Suvarna Talluri [a], Subramanian Mohan Raj [a,1], Lew Paul Christopher [a,b,*]

[a] Center for Bioprocessing Research and Development, South Dakota School of Mines and Technology, Rapid City, SD 57701, USA
[b] Department of Civil and Environmental Engineering, South Dakota School of Mines and Technology, Rapid City, SD 57701, USA

HIGHLIGHTS

- *Caldicellulosiruptor saccharolyticus* fermented untreated switchgrass and MCC to H_2.
- The extreme thermophile produced the maximum theoretical yield of 4 mol H_2/mol glucose.
- Pretreatment, enzyme production, hydrolysis, fermentation were combined in one step.
- *C. saccharolyticus* DSM 8903 is a promising candidate for consolidated bioprocessing.
- Potential for cost savings from capital and operating expenditures could exceed 50%.

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ABSTRACT

The abilities of the extreme thermophilic bacterium *Caldicellulosiruptor saccharolyticus* DSM 8903 to ferment switchgrass (SWG), microcrystalline cellulose (MCC) and glucose to hydrogen (H_2) in one-step were examined. Hydrogen production from glucose reached the theoretical maximum for dark fermentation of 4 mol H_2/mol glucose. The H_2 yield on MCC and SWG after 6 days of fermentation was 23.2 mmol H_2/L or 9.4 mmol H_2/g MCC and 14.3 mmol H_2/L or 11.2 mmol H_2/g SWG, respectively. The rate of H_2 formation however was higher on MCC (0.7 mmol/L h) than SWG (0.1 mmol/L h). *C. saccharolyticus* DSM 8903 was able to produce H_2 directly from mechanically-comminuted SWG without any physicochemical or biological pretreatment. Combining four processing steps (pretreatment, enzyme production, saccharification and fermentation) into a single biorefinery operation makes *C. saccharolyticus* DSM 8903 a promising candidate for consolidated bioprocessing (CBP) of lignocellulosic biomass.

1. Introduction

Lignocellulosic biomass is a renewable and abundant resource that is available in the U.S. at nearly 1.3 billion tons per year as a low-cost feedstock for production of biofuels (Zheng et al., 2012). However, due to the intimate association of polysaccharides and lignin, the lignocellulosic matrix is recalcitrant to enzymatic breakdown and pretreatment is usually required to facilitate the release of fermentable sugars from plant biomass. The conversion of cellulose and hemicellulose, in their cell wall-bound form, into fermentable sugars in a cost-effective way presents a major challenge to the commercialization of cellulosic biofuels. Several thermal, chemical, physical and biochemical pretreatment methods have been proposed to convert lignocellulosic biomass into fermentable sugars, but none of these approaches have been commercialized due to two major reasons: (1) high costs of pretreatment processes; and (2) generation of microbial growth inhibitory compounds such as phenols, organic acids, furfurals and/or hydroxylmethyl furfurals in most thermal and acid pretreatment processes (Yang and Wyman, 2008). The pretreatment process is usually energy-intensive and/or requires the use of hazardous chemicals and expensive enzymes for the hydrolysis step. Economic analyses have revealed that the greatest fraction of projected costs of nearly 40% is associated with the release of fermentable sugars from

* Corresponding author at: Center for Bioprocessing Research and Development, South Dakota School of Mines and Technology, Rapid City, SD 57701, USA. Tel.: +1 605 394 3385; fax: +1 605 394 1686.
E-mail address: lew.christopher@sdsmt.edu (L.P. Christopher).
[1] Present address: Center for Research and Development, PRIST University, Thanjavur, TN 613 403, India.

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biomass by the combined operations of pretreatment, enzyme production, and enzymatic hydrolysis, with pretreatment responsible for almost half of this total (Wooley et al., 1999). With 20% of the total costs, pretreatment is projected to be the single, most expensive processing and rate-limiting step (Yang and Wyman, 2008) of the overall H_2 production process. Hence, the key to the success of cellulosic H_2 relies on the development of breakthrough technologies allowing effective and low-cost saccharification and fermentation of lignocellulosic feedstock.

A significant step toward commercialization of cellulose-derived biofuels including H_2 is presented with the CBP process configuration which combines three major biomass processing steps – enzyme production, enzyme hydrolysis of the biomass carbohydrate components (cellulose and hemicellulose), and fermentation of the hexose and pentose sugars – into a single step (Lynd et al., 2005; Bielen et al., 2013). This integrated process has been proposed as the most cost-efficient and ultimate industrial configuration for low cost hydrolysis and fermentation of cellulosic biomass. CBP has an outstanding potential for cost savings in excess of 50% compared to other process configurations such as simultaneous saccharification and fermentation (SSF) or co-fermentation (SSCF) due to the elimination of the operating and capital costs associated with the additional step of enzyme production in the SSF and SSCF configurations (Olson et al., 2011). It has been estimated that the cost of ethanol produced by CBP is 4.5-fold lower than that of the SSCF configuration (Lynd et al., 2005).

CBP of lignocellulosic feedstock for H_2 production using thermophiles such as *Caldicellulosiruptor* sp. (Ivanova et al., 2009), *Clostridium* sp. (Levin et al., 2006), *Thermoanaerobacter* sp. (Shaw et al., 2010) and *Thermoanaerobacterium* sp. (Karadag et al., 2009) offers additional advantages in biomass processing over the use of mesophilic microbial systems (Mohan Raj et al., 2012). As the rate of biomass degradation increases with temperature, several techno-economical advantages to the process of H_2 can be realized through a thermophilic CBP: (1) close to theoretical maximum yield of 4 mol H_2/mol glucose; (2) utilization of a wide range of complex polymeric substrates; (3) favorable thermodynamics of stochiometric H_2 yields at higher temperatures; (4) diminished possibility of contamination by unwanted microorganisms that compete for the same substrates; (5) increased reaction/conversion rates due to improved mass transfer rates, improved substrate accessibility and solubility at elevated temperatures; (6) reduced formation of by-products; and (7) potential savings of capital and operating costs.

The extreme thermophile *Caldicellulosiruptor saccharolyticus* has been reported to produce H_2 on simple sugars such as fructose, arabinose, xylose, mannose, glucose, galactose (in descending order) (Van Fossen et al., 2009), and on a variety of polymeric substrates including agricultural waste such as wheat straw, bagasse, maize leaves (Ivanova et al., 2009), energy crops such as sweet sorghum (Ivanova et al., 2009; Panagiotopoulos et al., 2010) and *Miscanthus* (de Vrije et al., 2009), industrial waste such as paper sludge (Kadar et al., 2004), and food waste such as potato peels (Mars et al., 2010). However, no reports have been found in literature on utilization of SWG for H_2 production. The chemical composition of SWG varies with an average content of 34–39% glucan, 27–35% hemicellulose, and 19–23% lignin (Yan et al., 2010). The relatively high carbohydrate and low lignin contents makes SWG a very suitable biorefinery feedstock for bioprocessing to biofuels and biochemicals. Here we report on the direct utilization of SWG without a prior physicochemical or biological pretreatment for H_2 production by *C. saccharolyticus* strain DSM 8903.

2. Methods

2.1. Substrates and chemicals

Untreated SWG samples were a generous gift from Dr. K. Muthukumarappan from South Dakota State University, Brookings, SD. Prior to use, SWG was milled and sieved to obtain a fraction of 180 mesh uniform particle size referred to further in the text as "untreated switchgrass". MCC with an average particle size of 50 μM was purchased from Acros Organics (Thermo Fisher Scientific, NJ, USA). Yeast extract (Cat. 212750) was obtained from Difco (Becton Dickinson; Franklin Lakes, NJ, USA). Glucose and all other chemicals and reagents, unless otherwise indicated, were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Microorganism and culture conditions

The strain *C. saccharolyticus* DSM 8903 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection, Braunschweig, Germany. The strain was cultivated in DSM 640 culture medium with slight modifications. The culture medium had the following components per liter of deionized water: MCC, 2 g; NH_4Cl, 0.9 g; $MgCl_2{\cdot}6H_2O$, 0.4 g; NaCl, 0.9 g; $FeCl_3{\cdot}6H_2O$, 2.50 mg; K_2HPO_4, 1.5 g; KH_2PO_4, 0.75 g; yeast extract (YE), 1.0 g; cysteine-HCl, 0.75 g; resazurin, 0.5 mg; and trace element solution, 1.0 ml. The component trypticase of DSM 640 medium was replaced with casamino acid, 2.0 g/L. The trace element solution had the following components per liter of water: HCl (7.7 M), 10.00 ml; $FeCl_2{\cdot}4H_2O$, 1.50 g; $ZnCl_2$, 70.00 mg; $MnCl_2{\cdot}4H_2O$, 100.00 mg; H_3BO_3, 6.00 mg; $CoCl_2{\cdot}6H_2O$, 190.00 mg; $CuCl_2{\cdot}2H_2O$, 2.00 mg; $NiCl_2{\cdot}6H_2O$, 24.00 mg; $Na_2MoO_4{\cdot}2H_2O$, 36.00 mg. The initial pH was set at 7.2. Unless stated otherwise, the strain was grown anaerobically in 100 ml serum bottles containing 25 ml culture medium at 65 °C and 150 rpm in an orbital incubator shaker. The bottles were flushed with nitrogen gas (99.99%) for 15 min to ensure that the bottles were completely deprived of oxygen. The bottles were then sealed with butyl rubber septa and aluminum caps and autoclaved.

2.3. Determination of optimal substrates concentrations

The effects of initial SWG and glucose concentrations on the production of H_2 were studied in the range of 1–4% (w/v) in the DSM culture medium. In order to examine the effects of switchgrass and glucose, MCC of DSM medium 640 was replaced with either SWG or glucose as carbon source. The effect of yeast extract was studied in the range of 0.1–0.5% (w/v) by cultivating the strain in a modified DSM medium 640 containing 3% (w/v) SWG. In order to study the effects of insoluble SWG, the water-soluble extractives were removed from SWG by washing it in deionized water at 2% (w/v) with constant stirring at 70 °C overnight as described previously (Yang et al., 2009). The soluble extractives were filtered off and washed with deionized water twice at room temperature. The washed SWG was dried overnight at 60 °C and used in the H_2 production experiments. A seed culture of *C. saccharolyticus* DSM 8903 was routinely prepared in the modified DSM medium 640 containing 3% (w/v) SWG for 4 days. About 5 ml of a 4 day-grown culture broth was used as seed culture in all experiments. Samples were withdrawn periodically to determine cell density, residual substrate and metabolites concentration according to the methods described previously (Mohan Raj et al., 2009).