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Production of lactic acid from paper sludge using acid-tolerant, thermophilic *Bacillus coagulan* strains

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ABSTRACT

Production of lactic acid from paper sludge was studied using thermophilic *Bacillus coagulan* strains 36D1 and P4-102B. More than 80% of lactic acid yield and more than 87% of cellulose conversion were achieved using both strains without any pH control due to the buffering effect of $CaCO_3$ in paper sludge. The addition of $CaCO_3$ as the buffering reagent in rich medium increased lactic acid yield but had little effect on cellulose conversion; when lean medium was utilized, the addition of $CaCO_3$ had little effect on either cellulose conversion or lactic acid yield. Lowering the fermentation temperature lowered lactic acid yield but increased cellulose conversion. Semi-continuous simultaneous saccharification and co-fermentation (SSCF) using medium containing 100 g/L cellulose equivalent paper sludge without pH control was carried out in serum bottles for up to 1000 h. When rich medium was utilized, the average lactic acid concentrations in steady state for strains 36D1 and P4-102B were 92 g/L and 91.7 g/L, respectively, and lactic acid yields were 77% and 78%. The average lactic acid concentrations produced using semi-continuous SSCF with lean medium were 77.5 g/L and 77.0 g/L for strains 36D1 and P4-102B, respectively, and lactic acid yields were 72% and 75%. The productivities at steady state were 0.96 g/L/h and 0.82 g/L/h for both strains in rich medium and lean medium, respectively. Our data support that *B. coagulan* strains 36D1 and P4-102B are promising for converting paper sludge to lactic acid via SSCF.

1. Introduction

Lactic acid is an important industrial chemical with wide applications in the food, pharmaceutical, leather, and cosmetic industries (Datta et al., 1995; Garde et al., 2002; Lunt, 1998; Passos et al., 1994). It has drawn wide attention in recent years as a precursor for poly-lactic acid production that subsequently can be used for the production of biodegradable plastic that can replace plastic made from petroleum. In the United States, 90% of lactic acid production occurs via fermentation (Datta et al., 1995; Lunt, 1998), and starch-based materials are utilized exclusively as the feedstock (Datta et al., 1995; Patel et al., 2005). Lactic acid could potentially be produced from cheap and abundant cellulosic materials such as agricultural waste, municipal and industrial solid waste, and forestry waste (Lynd et al., 1991, 1999; Wyman, 2003). However, the process using cellulosic-based material is more complicated than that for starch-based material. First, the hydrolysate of cellulosic material contains five-carbon sugars and six-carbon sugars, both of which need be efficiently converted to lactic acid. Second, most cellulosic feedstocks require a complicated and expensive pretreatment process to hydrolyze hemicellulose into monomer sugars and to make the cellulose in those materials accessible to enzymatic hydrolysis (Lynd et al., 1999; Sheehan and Himmel, 1999; Wyman et al., 2005). Third, the cellulase enzymes needed to hydrolyze the pretreated cellulose to monomer sugars are expensive to produce (Castellanos et al., 1995; Jana et al., 1994; Lynd et al., 1999; Wooley et al., 1999; Wyman, 2003). Moreover, the enzymatic hydrolysis step is typically combined with the fermentative step to achieve simultaneous saccharification and fermentation (SSF) (Abe and Takagi, 1991; Wright, 1988; Yanez et al., 2003). If a strain that is able to ferment both five-carbon and six-carbon sugars is used, the process is called simultaneous saccharification and co-fermentation (SSCF) (Lynd et al., 1999; Patel et al., 2005). To achieve great efficiency in both SSF and SSCF, it is desirable to establish conditions optimal for two biocatalysts: cellulases and the microorganism (Patel et al., 2004; Wooley et al., 1999).

Lactic acid bacteria (LAB) are the most widely utilized microorganisms for lactic acid production (Carr et al., 2002; Litchfield, 1996; Stiles and Holzapfel, 1997). However, most LAB lack the ability to ferment xylose; except for a few species such as *Lactobacillus pentosus* and *Lactococcus lactis* (Hofvendahl and Hahn-Hagerdal, 2000). These type I *Lactobacilli* strains ferment xylose to lactic acid via a phosphoketolase pathway (Garde et al., 2002; Hofvendahl

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and Hahn-Hagerdal, 2000; John et al., 2007). One mole of xylose yields 1 mole of acetic acid and 1 mole of lactic acid. Hence the theoretical yield of lactic acid from xylose is about 60%. Producing acetate as a by-product of lactic acid fermentation requires extra work in the downstream process (Garde et al., 2002; Hofvendahl and Hahn-Hagerdal, 2000; Patel et al., 2006; Tanaka et al., 2002). In addition, the LAB widely used in industry requires rich media and fermentation conditions that are quite different from the optimal conditions for fungal cellulases (Carr et al., 2002; Hofvendahl and Hahn-Hagerdal, 2000; Wooley et al., 1998; Yanez et al., 2003).

Three new isolated thermophilic and acid-tolerant *B. coagulan* strains were reported to be very attractive candidates for SSCF for lactic acid production (Patel et al., 2004, 2005, 2006). These strains produce lactic acid as the major fermentation products from both glucose and xylose (Patel et al., 2006). Specifically, they produce lactic acid from xylose via the pentose phosphate pathway, through which 3 moles of xylose yield 5 moles of lactic acid. In other words, the theoretical yield of lactic acid from xylose is close to 100%, and no by-product is formed through this reaction (Patel et al., 2006). Moreover, these *Bacillus* strains grow optimally at 50 °C and pH 5.0, which coincide with the optimal conditions for commercial fungi-based cellulase enzymes (Patel et al., 2006).

In this paper we report the conversion of paper sludge to lactic acid using thermophilic *B. coagulan* strains 36D1 and P4-102B. In most paper mills, paper sludge – the solid waste product in the papermaking industry – is disposed of via land fill. However, paper sludge is a very attractive cellulosic feedstock for conversion to ethanol and other value-added products (Duff et al., 1994, 1995; Fan et al., 2003; Jeffries and Schartman, 1999; Katzen and Fowler, 1994; Lark et al., 1997). Compared to other cellulosic material, paper sludge has unique advantages, such as no need for costly pretreatment, negative feedstock cost, and potential integration into the current infrastructure (Fan et al., 2003; Fan and Lynd, 2007a,b; Lynd et al., 2001). Conversion of paper sludge to ethanol has been studied under industrially-like conditions, and conversions of more than 90% and ethanol concentrations of more than 40 g/L were achieved (Fan et al., 2003). However, paper sludge is a potentially advantageous feedstock for conversion to lactic acid due to its high content of $CaCO_3$ (up to 16% on a dry-weight basis) (Lynd et al., 2001), which is typically added as a buffering reagent to neutralize lactic acid and to maintain the pH of the fermentation system (John et al., 2007). Since paper sludge contains $CaCO_3$, it can potentially lower overall production costs by reducing the buffering reagent usage during the lactic acid production process. Conversion of paper sludge to lactic acid was studied and modeled in a fed-batch mode using a strain of *Lactobacillus rhamnosus* (Lin et al., 2005). However, the buffering effect of $CaCO_3$ was not studied. In this paper we describe the use of *B. coagulan* strains to convert paper sludge to lactic acid via SSCF using both lean and rich media and both batch and semi-continuous modes.

2. Methods

2.1. Sources of sludge, strains, and cellulase

The paper sludge used was primary clarifier sludge obtained from the paper mill in the Cascade mill of Pulp and Paper of America (Gorham, NH), which uses the bleached Kraft process. All paper sludge used for the experiments was stored at 4 °C upon arrival and was used unaltered. The *Bacillus* strains 36D1 and P4-102B were obtained from University of Florida, Gainesville. The cultures were stored at −80 °C, and a fresh batch was prepared for each experiment. Cellulase was kindly provided by Genencor International (Rochester, NY). All chemicals, including corn steep liquor, were purchased from Sigma Aldrich.

2.2. Batch and semi-continuous experiments

Batch experiments were conducted in 200 mL sealed serum bottles with a working volume of 100 mL and a N_2 gas phase as described by Lynd et al. (2001). Two growth media were used: a rich medium (Luria Broth) and a lean medium, each liter of which contains 1.36 g of KH_2PO_4, 2.0 g of NaCl, 0.2 g of $MgSO_4{\cdot}7H_2O$, 1.0 g of $(NH_4)_2SO_4$, 10 mg of $FeSO_4{\cdot}7H_2O$, and 0.5% corn steep liquor (wt/vol), as described in Patel et al. (2006). Paper sludge was added to the serum bottles containing either the lean medium or the rich medium until the cellulose content reached 20 g/L. The pH was adjusted to 5.0. Serum bottles containing the medium and the sludge were autoclaved at 121 °C for 20 min. Varied amounts of cellulase were added to produce the desired enzyme loading. Batch experiments were initiated by inoculating 1% of inoculum and incubating on a rotary shaker at 42 °C or 50 °C.

Semi-continuous experiments were conducted following procedures similar to those of Fan et al. (2003), using serum bottles instead of reactors. For the experiments, feeding frequency was two, and residence time was 4 days; that is, half of the reaction mixture was removed as products, and fresh paper sludge, enzyme, and medium equivalent to half the volume of the reaction mixture were added every 2 days.

The semi-continuous experiments started with an initial cellulose concentration of 40 g/L. The first sampling and feeding of serum bottles took place after 96 h of culture. Half of the reaction mixtures were removed and put into new serum bottles containing paper sludge equivalent to 100 g/L of cellulose, fresh enzyme and complementary medium to make the total volume equal to original volume. The procedure was repeated every 48 h. The residual reaction mixtures were taken as samples and analyzed for sugar and acid content. The working volume was 100 mL for all experiments, and all experiments were performed in duplicate under aseptic conditions. Because bottles were equipped with aluminum seals, the gas formed during the fermentation was released using a hypodermic needle at regular intervals. For the batch experiments, temperature, enzyme concentration, and the percentage of $CaCO_3$ were the parameters, while an enzyme loading of 15 FPU/g of cellulose and a temperature of 50 °C were used for semi-continuous experiments.

2.3. Sample analysis

The concentrations of sugars and organic acids in the samples were determined by HPLC. First, 1 mL of the sample was added to 0.1 mL of 10% H_2SO_4 and centrifuged at 1500*g* for 15 min. The supernatant was analyzed for sugars, lactic acid, and acetic acid using an Aminex HPX-87H column (Biorad, Hercules, CA) using the method described by South et al. (1995). The concentration of oligomer was analyzed using the method described by Fan et al. (2003). Samples were centrifuged at 1500*g* for 15 min. Nine-tenths milliliter of supernatant was added to 0.1 mL of 10% H_2SO_4. Samples were then transferred to 10-mL serum bottles, sealed, and autoclaved at 121 °C for 30 min. The resulting samples were analyzed using HPLC, as described above.

2.4. Calculations of conversion and lactic acid yields

Calculation of lactic acid yield was based on consumed glucose and xylose. Conversion was calculated based on cellulose using the equation presented in Lynd et al. (2001). The calculation of semi-continuous conversion and lactic acid yield followed the method described in Fan et al. (2003) using steady state data.