Journal of Biotechnology 144 (2009) 259–267

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Development of a high-throughput method to evaluate the impact of inhibitory compounds from lignocellulosic hydrolysates on the growth of *Zymomonas mobilis*

Mary Ann Franden, Philip T. Pienkos, Min Zhang*

National Bioenergy Center, MS3323, National Renewable Energy Laboratory, Golden, CO, 80401, USA

ARTICLE INFO

Article history:
Received 31 December 2008
Received in revised form 1 August 2009
Accepted 7 August 2009

Keywords:
Zymomonas mobilis
High-throughput screening
Cell growth assay
Bioscreen C
Cellulose
Hemicellulose
Hydrolysate
Biomass
Ethanol
Lignocellulosic

ABSTRACT

Overcoming the effects of hydrolysate toxicity towards ethanologens is a key technical barrier in the biochemical conversion process for biomass feedstocks to ethanol. Despite its importance, the complexity of the hydrolysate toxicity phenomena and the lack of systematic studies, analysis and tools surrounding this issue have blocked a full understanding of relationships involving toxic compounds in hydrolysates and their effects on ethanologen growth and fermentation. In this study, we developed a quantitative, high-throughput biological growth assay using an automated turbidometer to obtain detailed inhibitory kinetics for individual compounds present in lignocellulosic biomass hydrolysate. Information about prolonged lag time and final cell densities can also be obtained. The effects of furfural, hydroxymethylfurfural (HMF), acetate and ethanol on growth rate and final cell densities of *Zymomonas mobilis* 8b on glucose are presented. This method was also shown to be of value in toxicity studies of hydrolysate itself, despite the highly colored nature of this material. Using this approach, we can generate comprehensive inhibitory profiles with many individual compounds and develop models that predict and examine toxic effects in the complex mixture of hydrolysates, leading to the development of improved pretreatment and conditioning processes as well as fermentation organisms.

1. Introduction

Lignocellulosic materials such as agriculture residues, woody biomass and herbaceous plants are an abundant and renewable source for ethanol fuel production by fermentation. These cellulosic materials contain up to 70% of their dry mass as structural carbohydrates (cellulose and hemicellulose) and are significant feedstock sources for biofuels and chemical production. However, unlike starch, currently the primary source for fuel ethanol, the carbohydrates in biomass are closely associated with lignin in the plant cell wall, and pretreatment using a thermo and/or chemical process is necessary to make them available for enzymatic hydrolysis and fermentation. Although pretreatment processes are designed to breakdown the cellulose, hemicellulose and lignin matrix, releasing monosaccharides and making the remaining polysaccharides available for enzymatic saccharification, they often result in production of toxic compounds which inhibit subsequent microbial fermentation. The toxic nature of lignocellulosic hydrolysate results in increased process costs due to additional detoxification steps or constraints on solids loading in the fermentation step (Aden and Foust, 2009). The dilute acid pretreatment process gives rise to organic acids, primarily acetic acid, sugar degradation products such as furfural and hydroxymethylfurfural (HMF), phenolics from lignin degradation as well as inorganic salts mainly arising from the pretreatment process. Recently, extensive reviews were conducted on inhibitors formed by pretreatment of lignocellulosic materials and their inhibition of ethanol production in yeast and bacteria (Pienkos and Zhang, 2009; Klinke et al., 2004; Palmqvist and Hahn-Hagerdal, 2000a, 2000b). Microorganisms differ in their ability to grow in hydrolysates and related inhibitory compounds, and the fermentative performances of microorganisms in lignocellulosic hydrolysates also depend on biomass feedstocks and pretreatment conditions (see for example, Zaldivar and Ingram, 1999; Zaldivar et al., 1999, 2000). Several methods have been used for evaluating and comparing toxicity on microbial fermentation which include cell growth, ethanol yield and ethanol productivity. Because so many variables have been considered (methods used for toxicity measurement, fermentation organisms, inoculum levels, biomass feedstock, pretreatment conditions, and choice of model toxic compounds), the analyses of toxicity by hydrolysates and model compounds in the literature are often difficult to compare properly.

Cell growth is associated with ethanol production by *Saccharomyces cerevisiae*, recombinant *Escherichia coli* and *Zymomonas*

* Corresponding author. Tel.: +1 303 384 7753; fax: +1 303 384 7752.
E-mail address: min.zhang@nrel.gov (M. Zhang).

0168-1656/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2009.08.006

mobilis. Inhibition of cell growth has been shown to be strongly related to inhibition of ethanol production for many inhibitory compounds (Zaldivar and Ingram, 1999; Zaldivar et al., 1999, 2000; Taherzadeh et al., 1999; Palmqvist et al., 1999; Helle et al., 2003). It is used widely to evaluate the toxicity of various inhibitor compounds on microbial fermentation. However, the parameters used to evaluate inhibition varied widely making it difficult to assess whether inhibition relates to growth or fermentation or both. Growth inhibition on glucose does not necessarily reflect overall fermentation on mixed substrates. A closer examination of inhibition on growth and fermentation on respective substrates is needed. Measurement of cell viability and growth provide reliable and sensitive assays for characterization of toxic compounds and conditions that adversely affect microbial cells. However, the execution of individual growth-associated assays is slow and laborious. Typical literature studies report relative growth measured as percentage of growth of the control, cultured in the absence of inhibitor, using a single end point (See for example Delgenes et al., 1996). In addition, traditional growth assays frequently yield categorical data rather than the discrete quantitative data that can provide a solid basis to examine inhibitory mechanisms and prioritize toxicities of the individual compounds.

Understanding the toxic effects on cell growth and fermentation is critical to elucidate toxicity mechanisms and to allow development of pretreatment processes that reduce inhibitory compound formation along with the potential development of more process-robust ethanologens and improvement of fermentation processes.

To obtain the comprehensive inhibition profile of various compounds found in hydrolysates, we developed a quantitative, high-throughput biological growth assay using the Bioscreen C instrument, an automated turbidometer using *Z. mobilis* 8b as the test organism. Several common inhibitors found in hydrolysate, such as acetate, HMF, furfural, and ethanol were tested using this method. In addition, this method is shown to be applicable for evaluating toxicity of hydrolysates despite the highly colored nature of these liquids which can interfere with cell mass estimations that are based on optical densities.

2. Materials and methods

2.1. Reagents and strains

The two *Z. mobilis* strains used in this study were wild-type ZM4 (ATCC 31821) and recombinant strain 8b (Zhang et al., 2008). Cultures were grown in RM medium (10 g l^{-1} yeast extract, 2 g l^{-1} KH_2PO_4) supplemented with 2% (w/v) glucose (RMG) for inhibitor studies, pH 5.8. Hydrolysates were supplemented with RM medium and sugars to a total final concentration of 2% glucose and 4% xylose, with pH adjusted to 5.8 with phosphoric acid. Strain 8b was also cultured in RM medium supplemented with 2% (w/v) xylose (RMX) for correlating linear and non-linear cell density studies. Glacial acetic acid was obtained from JT Baker, and stocks of 100 g l^{-1} were prepared in water and pH adjusted with 10N KOH to 5.8. Hydroxymethylfurfural (5-hydroxymethyl-2-furaldehyde, HMF) (99% purity) was purchased from Aldrich and prepared as 100 g l^{-1} stock in water. Furfural (2-furaldehyde) (99% ACS grade purity) was supplied from Sigma. Because of its low solubility, furfural stocks were prepared directly in media at 3.6 g l^{-1}. Ethanol (from AAPER Alcohol and Chemical, Co, 200 proof) stocks were also prepared in RMG at 100 g l^{-1} concentration, and dilutions made thereof. The pH of all medium prepared was 5.8. All media and reagents were filter sterilized.

Table 1
Concentrations of compounds (g l^{-1}) in pre-treated corn stover liquor (PCS) and in NH4-conditioned hydrolysates (NH4-cond hydrolysate).

Compound (g l^{-1})	PCS	NH4-cond hydrolysate
Cellobiose	2.9	2.7
Glucose	29.3	29.4
Xylose	80.7	81.3
Galactose	5.9	6.0
Arabinose	13.0	13.2
Fructose	4.3	4.4
Lactic acid	1.0	0.7
Glycerol	0.8	0.6
Acetic acid	15.9	15.6
HMF	3.1	1.9
Furfural	1.8	1.2

2.2. Corn stover hydrolysates

The pretreated hydrolysate used in this work was produced from milled corn stover harvested in the fall of 2003 from the Kramer farm in Wray, Colorado. The stover was pretreated with dilute sulfuric acid in the 900 dry kg d^{-1} pilot-scale vertical continuous reactor in the NREL Process Development Unit (PDU). Pretreatment operating conditions included an insoluble solids loading of 30% (w/w), a sulfuric acid loading of 0.048 g acid/g dry biomass, an approximate residence time of 1 min, and a reaction temperature of 190 °C (Jennings and Schell, 2006). Pretreated hydrolysate was obtained through solid liquid separation of the whole pretreatment slurry via use of a hydraulic press operated at approximately 2000 psig, which removed approximately 70% of the hydrolysate from the pretreated slurry.

2.3. Conditioning of hydrolysate

The hydrolysate obtained from the solid–liquid separation step described above was subjected to a conditioning processes using ammonium hydroxide (29.8% assayed as NH_3, J.T. Baker, USA) as described by Jennings and Schell (2006). After increasing the pH up to 8.5 with 29.8% ammonium hydroxide (50 ml l^{-1}), the temperature rose ~10 °C due to addition of base from room temperature and held at 30 °C for 30 min. Hydrolysates were then filtered, and pH re-adjusted to 6.8 using concentrated sulfuric acid (10N, J.T. Baker, USA) then filtered a second time.

2.4. HPLC analysis

Concentrations of ethanol, HMF, furfural, lactic acid, glycerol, and acetic acid present in hydrolysates were determined from filtered sample supernatants by high performance liquid chromatography (Agilent1100 series, Agilent USA, Santa Clara, CA) utilizing a BioRad HPX-87H organic acids column and Cation H^+ guard cartridge (BioRad Laboratories, Hercules, CA) operating at 55 °C. A refractive index detector was used for compound detection. Dilute sulfuric acid (0.01N) was used as the isocratic mobile phase at a flow rate of 0.6 ml min^{-1}. Mixed component concentration verification standards were periodically run with the HPLC samples to verify calibration accuracy. Sugars including glucose, xylose, fructose, mannose, galactose, arabinose and cellobiose were measured by high performance liquid chromatography (Agilent 1100 series) using a Shodex SP0810 carbohydrate column with de-ashing guard cartridges (BioRad Laboratories, Hercules, CA) run at 85 °C with ultra-pure water as the isocratic mobile phase at a flow rate of 0.6 ml min^{-1}. The concentrations of sugars, acids and furfurals in pre-treated corn stover hydrolysate (PCS) as well as ammonium conditioned hydrolysate used in this study are presented in Table 1.