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On-line Monitoring of Microbial Fermentation for Cellulosic Biofuel Production

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ABSTRACT Bioprocesses are regarded as a potentially sustainable method for the production of biofuels and other value-added co-products. However, most bioprocesses cannot yet compete with conventional technologies and require intensive research and development efforts. On-line analysis can help to facilitate dynamic study of microbial metabolism, optimal process control, and product quality assurance. Titration and Off Gas

Analysis (TOGA) is an on-line sensor that combines a membrane inlet mass spectrometer (MIMS) with on-line titration techniques to obtain real-time data of metabolic end products to reveal key information of the process state. The instrument is here applied to quantify volatile metabolic end products from fermentation of cellobiose with *Clostridium thermocellum*, including ethanol, CO_2, and H_2 in real time. Concurrent off-line analysis was performed for verification. Errors with respect to on-line analysis were found to be 4.8 ± 3.7% and 7.1 ± 3.5 %, 10.7 ± 9.0% for H_2, CO_2, and ethanol, respectively. Carbon and electron balances showed no significant differences and were close to theoretical values for both on-line and off-line data. The titration system effectively maintained the process pH at 7.2 ± 0.02 and also measured the hydrogen ion production (HP), which could be 95.9 ± 2.3% accounted for in the end product synthesis patterns, determined off-line. The sensor was shown to be a viable on-line sensor that can contribute dynamic insight into the development and control of bioprocesses for cellulosic biofuel production processes.

Keywords: on-line analysis, bioprocess control, membrane inlet mass spectrometer, biohydrogen, bioethanol, *Clostridium thermocellum*, titration, off-gas analysis

INTRODUCTION With uncertainties surrounding future petroleum supply, the opportunity exists for the commercial production of renewable alternative energy technologies. Microbial fermentation of waste biomass residues for the production of fuels and other value-added co-products offers great potential as an environmentally benign, sustainable alternative to displace fossil fuel dependence (Youngs and Somerville 2012). Biofuels of this type, known as third-generation, are advantageous for a number of reasons: 1) biological conversion platforms eliminate high temperatures and pressures required in thermochemical processes, and nearly theoretical yields can be achieved (Damartzis and Zabaniotou 2011; Wyman et al. 2005; Youngs and Somerville 2012); and 2) use of waste ligno-cellulosic residues from agriculture, forestry, and other industries is an inexpensive, readily available resource that is regarded as the only foreseeable source of sustainable energy (Levin et al. 2007; Lynd et al. 2002).

Clostridium thermocellum is a strictly anaerobic, gram positive, cellulolytic, thermophile that exhibits the ability to rapidly hydrolyze cellulose and ferment the resulting cellodextrans into a variety of metabolites that are of interest as chemical commodities, including biofuels (Carere et al. 2008; Islam et al. 2006). The predominant end products of fermentation include H_2, CO_2, ethanol, and acetate (Ellis et al. 2012; Islam et al. 2006; Levin et al. 2006; Rydzak et al. 2009). Formate and lactate synthesis have also been reported under certain growth conditions (Islam et al. 2006; Levin et al. 2006; Sparling et al. 2006). Despite the rapid rate of cellulose degradation exhibited by this organism, yields remain too low to be a commercially viable technology (Levin et al. 2006). According to Alford (2006) establishing appropriate monitoring and control strategies is necessary for bioprocess development.

Highly-precise, off-line analytical methods are usually employed for quantitative analysis of bioprocess variables. Typically, these techniques require extensive sample preparation followed by a lengthy analysis. This causes a time delay in bioprocess state estimation, making process control and optimization difficult (Gapes et al. 2003; Vojinovic et al. 2006) A real-time data profile of end product synthesis patterns from microbial fermentation can be a powerful tool for understanding metabolism dynamically, allowing implementation of

effective real-time process control schemes to optimize process efficiency, improve reproducibility, and reduce costs (Clementschitsch and Bayer 2006; Dietzsch et al. 2013; Vojinovic et al. 2006).

Titrimetric Off Gas Analysis (TOGA) is an on-line multi-analyzer system that couples an on-line titration system to a membrane inlet mass spectrometer (MIMS) and a bioreactor. The instrument has been previously used for monitoring biological wastewater treatment processes (Gapes et al. 2003; Pratt et al. 2003). There are several advantages of using MIMS as an on-line analytical instrument, including selectivity, sensitivity, rapid response, and ability to simultaneously monitor multiple analytes (Johnson et al. 2000). Titration is an analytical technique that studies biochemical and physicochemical processes in which the pH is affected (Pratt et al. 2004). Titration techniques have also been used for on-line analysis of volatile fatty acids, bicarbonate, and hydrogen ion production (Bouvier et al. 2002; Gapes et al. 2003; Pratt et al. 2003).

The research objectives of this paper are to use TOGA for the on-line monitoring of a thermophilic, pure-culture fermentation of cellobiose with *Clostridium thermocellum*. The MIMS will be used for detection of CO_2 and H_2 in the off-gas stream, and ethanol in the liquid phase. The titration system will be used for precise control of the process pH, and monitoring the hydrogen ion production (HP). The results will be verified with off-line analysis via gas chromatography (GC) and high-performance liquid chromatography (HPLC). The long-term objectives of this research are to use TOGA to gain dynamic insight of microbial metabolism, including rapid detection of metabolic shifts, and how external parameters affect internal biochemical pathways.

MATERIALS AND METHODS

Microorganism, substrates and media preparation The medium used for this study was 1191, a complex medium that has been previously described elsewhere (Islam et al. 2006). All required chemicals for media preparation were purchased from Fisher Scientific, with the exception of yeast extract and potassium phosphate dibasic, which were purchased from Sigma Chemical Co. (St. Louis, MO). Sterile, anaerobic cellobiose was added as a carbon source to all cultures to a final concentration of 2 g L^{-1}. The organism of study is *C. thermocellum* DSM 1237.

Bioreactor Setup A 7 L glass, single-wall, round-bottom bioreactor was used (Applikon Biotechnology, Foster City, CA). A volume of 2610 mL 1191 media (Islam et al. 2006) was added to the reactor prior to being autoclaved at 121°C for 60 minutes. The reactor was then purged overnight with an inert carrier gas consisting of 95% N_2 and 5% Ar (Welder's Supplies, Winnipeg, MB) at a flow rate of 10 mL min^{-1}. This setup was left overnight to establish anaerobic conditions. A 200 mmol L^{-1} solution of di-sodium sulfide (Na_2S) was then filter sterilized through the gas sampling port on the reactor head plate on a 1% (v/v) basis just prior to inoculation. Similarly, cellobiose was filter sterilized through the gas sampling port to a concentration of 2 g L^{-1}. The reactor was then inoculated (10% v/v) from a 1 L bottle that had been incubated for a 12-hour period.

On-line analytical methods MIMS calibration and operation An HPR-40 dissolved-species membrane inlet mass spectrometer equipped with a HAL 201 RC quadrupole mass

analyzer, Faraday Cup detector and Scanning Electron Multiplier (SEM) detector (Hiden Analytical, Warrington, UK), was used in conjunction with MasSoft 7® software to measure ethanol, H_2, and CO_2 on-line. For gas-phase calibration, a four-point background-subtracted concentration calibration curve was developed for the MIMS for 0-10% H_2 and 0-5% CO_2 (mol/mol). Known concentrations of these gases were prepared in a step-wise fashion by diluting purchased gas mixtures (Welder's Supply, Winnipeg, MB) into an inert carrier stream using thermal mass flow controllers (Bronkhorst Hi-Tech, El-flow, The Netherlands). The combined calibration gas mixture was then humidified to the same level water vapor level seen in the condensed reactor off-gas stream, prior to being measured at room temperature with the MIMS. Hydrogen was measured at a mass-to-charge ratio (m/z) of 2 and CO_2 was measured at m/z 22. For liquid phase analysis, ethanol calibration was carried out by the standard addition method, in a separate, anaerobic bottle containing 150 mL 1191 at 60C. A solution of 1 M ethanol was prepared and added to the vessel to final concentrations of 0.5, 1, 2, 5, 10, and 15 mM. The MIMS probe was used to measure the ethanol signal at m/z 31 and was allowed to equilibrate for five minutes at each concentration step. The background subtracted ion current for each signal of interest was then fitted to the known concentration using a linear regression analysis. All regression analysis was performed after fermentation within Microsoft Excel®.

Titration system calibration and operation The pH control system (Scion, Rotorua, New Zealand) utilizes two, high-precision 10 mL injector syringes (Hamilton Syringe Co) operated by split PID logic administered by a programmable logic controller (PLC, Opto22). The proportional gain was set to 10, the integral gain to 0.3 and the derivative term to zero. The update rate was set at 1 s^{-1}, and the dead band about the set point was set at 1% of the process variable range. The syringes are driven by an actuator to dose acid and base. The actuator breaks each stroke of the syringe into 100,000 pulses. The unit was calibrated by measuring the mass of 1 M HCl and 1M KOH dosed per 1,000 pulses and relating this to volume.

Fermentation experiments Following inoculation of the reactor, the conditions were maintained at 60°C, pH 7.2, and gentle stirring at 100 rpm was applied. During fermentation, the MIMS was used to either monitor H_2 and CO_2 in the off-gas stream at m/z 2 and m/z 22, respectively, or ethanol in the liquid-phase at m/z 31 until growth ceased. Simultaneously, samples were taken hourly for off-line analysis of both the gas phase and the liquid phase.

Off-line analytical methods Gas Phase An Agilent 7890A Gas Chromatograph System (Agilent Technologies Canada Inc. Mississauga, ON) equipped with a thermal conductivity detector (TCD) and a PLOT molecular sieve column 30 m by 0.53 m ID was used to verify the concentration of non-condensable gasses (H_2, CO_2) in the reactor off-gas detected by the MIMS.

Liquid Phase Liquid samples were processed according to the protocol in Ramachandran et al. (2008). Cellobiose, glucose, lactate, formate, acetate, and ethanol were quantified by an HPLC with a Model 1515 pump, Model 2707 autosampler, and a Model 2414 refractive index detector (Waters, Milford, MA). A 300mm x 7.8 mm resin-based column was used for separation (Aminex HPX-87H, Bio-Rad Laboratories, Mississauga, ON).

Solid Phase Pellets were processed as previously described (Ramachandran et al. 2008). Protein content was determined with the Bradford Assay (Bradford 1976). The protein data was used to account for cell mass in the carbon balance, approximating biomass as having the composition $C_4H_7O_2N$, and having a molecular weight of 101 g mol^{-1} (Rydzak et al. 2009).

Total Gas production A gas-phase mass balance was performed, accounting for venting of H_2 and CO_2 in the off-gas stream at a purge rate of 10 mL min^{-1}, by integrating the gas concentrations with respect to time using the trapezoidal rule. Total gas production was then found by adding the dissolved species at a given time. The concentration of H_2 and CO_2 remaining in solution was related to the headspace gas composition through Henry's Law. Temperature-corrected Henry's coefficient for each gas was determined from literature values (Sander 1999); the coefficient used for H_2 and CO_2 were 7.98×10^{-4} and 3.84×10^{-2} mol L^{-1} atm^{-1}, respectively. Carbon dioxide presents a unique challenge, as the molecule is associated with several dissolved carbonate species. These were accounted for using an approach previously described (Yang et al. 2003). The equilibrium constants used were $K_1 = 4.46 \times 10^{-7}$ and $K_2 = 4.69 \times 10^{-11}$ (Stumm and Morgan 1996).

Efficacy of results A mass balance and electron balance were used to determine the efficacy of both on-line and off-line results, indicating that all major end products had been accounted for and measured with adequate accuracy. The carbon recovery was calculated as the ratio of the carbon measured in the end products and cell mass to carbon utilized in the substrate, following Ellis et al. (2012). The electron balance was calculated as the ratio of oxidized end products to reduced end products.

RESULTS AND DISCUSSION

Gas Phase The composition of the condensed reactor off-gas composition was monitored continually with the MIMS, and in hourly intervals with the GC for verification. The data for total gas production, derived from on-line gas measurements with MIMS and off-line measurements with GC are shown in Figure 1. The total gas production accounts for solubility of the gases in the liquid phase and associated carbonate species, in the case of CO_2. This was done because off-gas measurements alone indicate the liquid-to-gas mass transfer rate, but the total production is a more accurate estimate of the actual biological production (Pratt et al. 2003). As indicated in Figure 1, the on-line and off-line measurements are in close agreement. The validation errors from measurements of total gas production after the start of growth for all biological replicates were found to be 4.8 ± 3.7% for H_2 and 7.1 ± 3.5 % for CO_2. The response of the MIMS during calibration proved to be linear for the range of concentrations tested, with all regression coefficients exceeding 0.99.

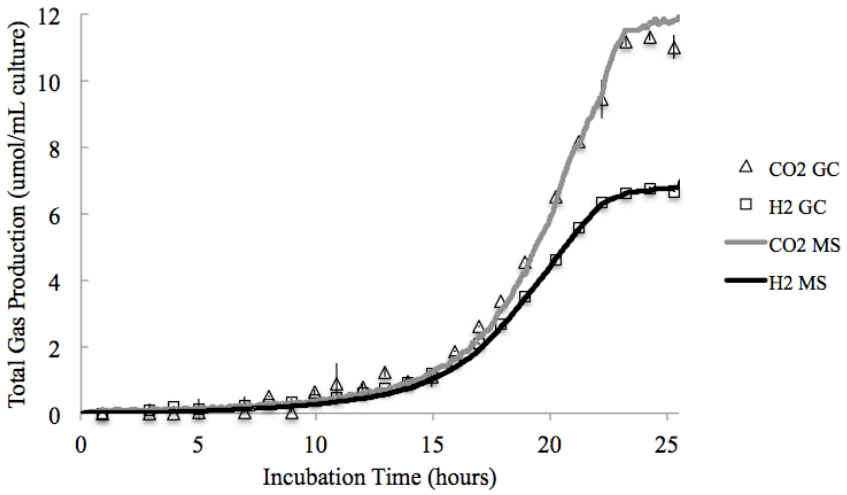

Figure 1. On-line and off-line data for total gas production.

Liquid Phase The major soluble end products determined by off-line HPLC include, in order of decreasing carbon flux, ethanol, acetate, and formate. Lactate production was variable, accounting for as much as 16.5% of the carbon flux in some instances, and not being detectable in others. Islam et al. (2006) found that lactate production increased to capture up to 18% of carbon flux as cells approached stationary phase and was accompanied by a pH drop below 6.7. However, for identical growth conditions, Rydzak et al. (2009) reported no lactate production. When the MIMS probe was devoted to analysis of the liquid phase ethanol was measured for comparison with HPLC results. This correlation is shown in Figure 2, where it is seen that the on-line MS and off-line HPLC measurements are in agreement with one another, with the average margin of error being 10.7 ± 9.0%. The majority of this error resulted from variable HPLC measurements made early in the growth period. The response of the MIMS proved linear for the range of concentrations tested, with all regression coefficients exceeding 0.99.

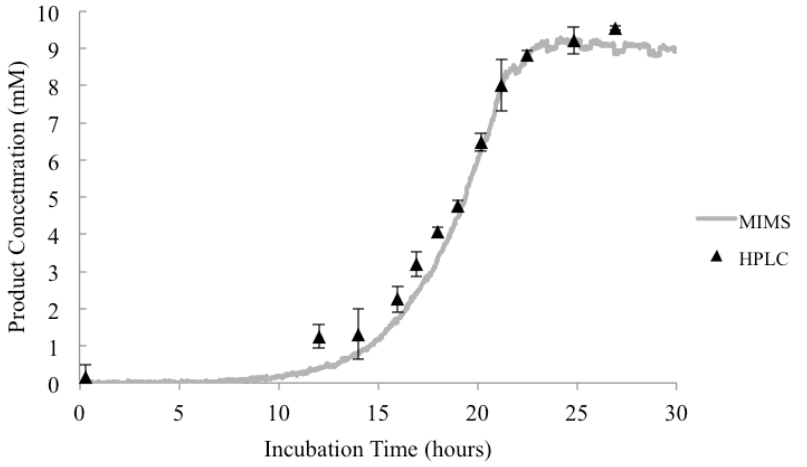

Figure 2. On-line and off-line data for ethanol analysis.

Titration Analysis The titration system served the dual purpose of maintaining the process pH and recording cumulative reagent addition. Because pH was maintained by the action of a split PID controller, small quantities of 1 M HCl acid were added with a second injector syringe to correct any overshoot from base addition. This creates a sinusoidal pattern about the set point in Figure 3, which shows the process pH and base addition throughout the fermentation process. Despite these small deviations, the controller was able to effectively maintain pH at 7.2 ± 0.02 throughout the course of fermentation, preventing growth arrest resulting from excessive pH drop.

The volumetric addition of acid and base addition was recorded to calculate the hydrogen ion production (HP). The HP is a function of the carbonate buffer system and production of organic acids. While CO_2 was measured with the MIMS, the organic acids could not be measured *in-situ* with the MIMS, because the process pH was greater than the dissociation constant of the acids produced, leaving them in their ionized, non-volatile form. As verification that the HP accurately reflects both acid production and CO_2 production, CO_2 data generated from the MIMS combined with HPLC data on organic acid production was used to perform a proton balance. Carbon dioxide, acetate, formate, and lactate were treated as monoprotic acids, and it was assumed that they dissociate completely at pH 7.2. On average, 95.9 ± 2.3% of the HP measured with the titration system by base addition could be accounted for by this method in each replicate (Table 1). The remainder that was unaccounted for may be due to small amounts of extra-cellular pyruvate, malate, and amino acids, which collectively have been shown to account for 4.1% of carbon flux (Ellis et al. 2012), and were not accounted for in this work. Regardless, this shows that, combined with reliable CO_2 data, the HP could be a metric to estimate organic acid production.

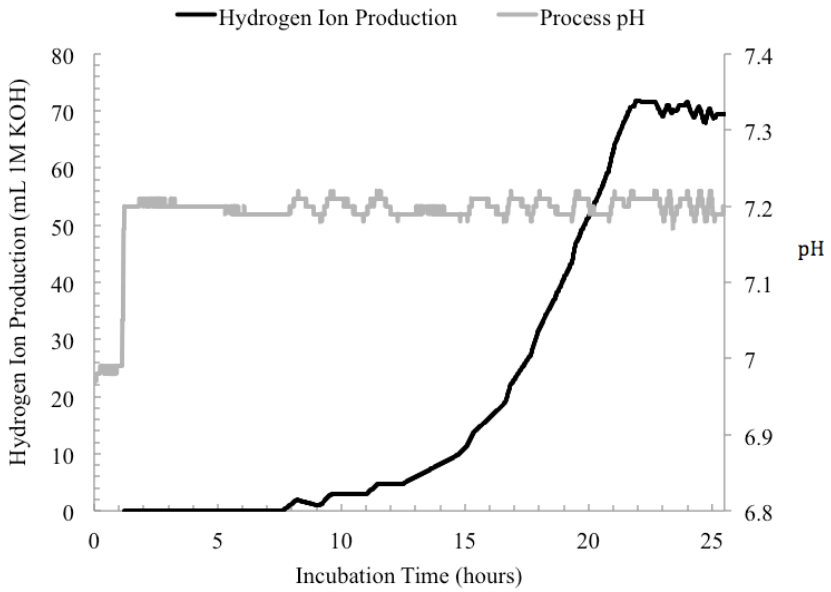

Figure 3. On-line pH control about the set point of 7.2 as well as hydrogen ion production, throughout the course of fermentation. The sudden jump in pH at 1 h corresponds to the time when the controller was first elicited.

Table 1. Hydrogen ion recovery from titration system for three biological replicate experiments expressed with respect to the theoretical hydrogen ion production given the end product profile.

Biological Replicate	Net addition of 1 M KOH	Total CO_2 production	Total lactate production	Total formate production	Total acetate production	Proton recovery
	(mL)	mmol	mmol	mmol	(mmol)	(%)
1	62.0	32.4	5.3	5.7	17.7	98.5
2	70.2	35.4	0.4	10.1	20.2	94.3
3	68.3	35.1	1.7	11.8	19.7	94.8
Mean						**95.9 ± 2.3**

Efficacy of Results The accuracy of both on-line and off-line analyses was reflected in the carbon recovery and electron balance. Together with off-line data, the gas data from both on-line data and off-line data were used to calculate these indices for all biological replicate experiments (Table 2). The results were found to be in close agreement. This suggests that all major end products have been adequately accounted for, and demonstrates that on-line analysis with MIMS does not compromise the accuracy of measurements.

Table 2. Comparison of off-line and on-line analysis for carbon recovery, and electron balance

Replicate	Carbon Recovery		Electron Balance	
	On-line	Off-line	On-line	Off-line
1	0.85 ± 0.13	0.85 ± 0.13	1.23 ± 0.13	1.24 ± 0.13
2	0.96 ± 0.20	0.97 ± 0.21	0.93 ± 0.19	1.06 ± 0.27
3	0.97 ± 0.17	0.93 ± 0.15	1.14 ± 0.08	1.10 ± 0.31
Mean	**0.93 ± 0.07**	**0.92 ± 0.15**	**1.10 ± 0.15**	**1.13 ± 0.09**

In comparison with similar studies using MIMS for on-line bioprocess analysis, Bastidas-Oyanedel et al. (2010) examined calibration strategies for H_2, CO_2 and ethanol in the gas and liquid phase for on-line analysis on anaerobic fermentation processes. This study found that standard addition calibration methods in which sterile standards were added to water in an N_2 headspace produced unacceptable levels of error in comparison with off-line methods. Instead it was recommended that calibration be performed by an in-process method, in which MIMS signals were calibrated to concurrent, matrix-free, off-line analysis. By applying the in-process calibration method to batch fermentation under mesophilic conditions, the latter method, errors ranged from 3 ± 1.5%, 0.03 ± 3%, and 11 ± 8% for CO_2, H_2, and ethanol, respectively (Bastidas-Oyanedel et al. 2010). In this study, we report similar errors for ethanol in the liquid phase and H_2 in the gas phase, and slightly higher level of error in the gas phase CO_2 measurements for the calibration methodology described which is independent of off-line methods.

CONCLUSIONS In this work, a TOGA sensor has been applied for the on-line study of a thermophilic pure-culture fermentation of cellobiose with *C. thermocellum*. The results for on-line analysis of the gas-phase target compounds H_2 and CO_2 were found to be in agreement with off-line GC analysis. Similarly, the on-line liquid phase analysis of ethanol with MIMS produced results that were consistent with off-line HPLC measurements. Regardless of whether on-line or off-line data was used, the carbon recovery and electron balance showed no significant difference, and were close to theoretical values. Furthermore, it was demonstrated that the HP measured by the titration system was in good agreement with off-line data for CO_2 and organic acids. This demonstrates that the HP signals from the titration system could be used as a metric for quantifying organic acid production and get additional information on the process state in real-time. Overall, the sensor has proven a viable on-line sensor that can contribute dynamic insight into bioprocess monitoring, control, and development.

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