Bioresource Technology 172 (2014) 112–120

Contents lists available at ScienceDirect

Bioresource Technology

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

Monitoring lignocellulosic bioethanol production processes using Raman spectroscopy

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HIGHLIGHTS

- Raman spectroscopy can enable *in situ* monitoring of biorefinery processes.
- Most promising is real-time measurements of the bioethanol fermentation.
- Quantification model accounts for disturbance from light scattering debris.
- Improved measurements achieved through partial precipitation of particulates.

ARTICLE INFO

Article history:
Received 19 April 2014
Received in revised form 14 August 2014
Accepted 16 August 2014
Available online 23 August 2014

Keywords:
Raman spectroscopy
Lignocellulose
Pretreatment
Hydrolysis
Ethanol fermentation

ABSTRACT

Process control automation in the emerging biorefinery industry may be achieved by applying effective methods for monitoring compound concentrations during the production processes. This study examines the application of Raman spectroscopy with an excitation wavelength of 785 nm and an immersion probe for *in situ* monitoring the progression of pretreatment, hydrolysis and fermentation processes in the production of lignocellulosic ethanol. Raman signals were attenuated by light scattering cells and lignocellulosic particulates, which the quantification method to some degree could correct for by using an internal standard in the spectra. Allowing particulates to settle by using a slow stirring speed further improved results, suggesting that Raman spectroscopy should be used in combination with continuous separation when used to monitor process mixtures with large amounts of particulates. The root mean square error of prediction (RMSE) of ethanol and glucose measured in real-time was determined to be 0.98 g/L and 1.91 g/L respectively.

1. Introduction

Concerns about increasing carbon dioxide emissions and the depletion of the remaining global oil reserves provide ample reasons to develop alternatives to fossil fuels. A promising renewable resource for the production of fuel for transportation exists in the form of bioethanol produced from the carbohydrates present in lignocellulosic biomass from abundant agricultural residues (Menon and Rao, 2012). The production of 2nd generation bioethanol involves some sort of pretreatment of biomass at elevated temperatures followed by enzymatic hydrolysis to release the sugars bound in cellulose and hemicellulose for the subsequent fermentation process. These processes can be operated more efficiently or even automated if production parameters can be monitored real-time instead of retrieving vital process information through manual time-consuming sampling and subsequent off-line analysis.

Various forms of absorption spectroscopy technologies present alternative *in situ* analysis methods to the commonly used high performance liquid chromatography (HPLC) or gas chromatography (GC) for measuring concentrations of multiple reaction components simultaneously (Bakeev, 2005; Beutel and Henkel, 2011). Near-infrared (NIR) and mid-infrared (MIR) spectroscopy have been used to monitor the fermentation processes (Finn et al., 2006; Mazarevica et al., 2004; Sivakesava et al., 2001). However, these analysis techniques are complicated by the broad overlapping peaks seen in NIR spectra or by the strong absorption of water in the MIR spectral range, unless used with an attenuated total reflection unit (ATR). In contrast, Raman spectroscopy is not based on absorption but on inelastic scattering of monochromatic light with a wavelength of the reemitted photons determined by

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http://dx.doi.org/10.1016/j.biortech.2014.08.068
0960-8524/

the Stokes energy shift of the molecule (Bakeev, 2005; Stewart et al., 1995). The need for a probe with a fixed light path within a gap is circumvented if the spectroscopy method is not based upon light transmission, since the same lens can be used for both the excitation light beam entering the sample and collecting the backscattering Raman signals. This makes Raman spectroscopy capable of analyzing viscous reaction mixtures or ones with high solids content. Raman spectra generally have distinct narrow peaks and the interference from water in aqueous samples is weak, unlike MIR. However, Raman signals are very weak and the laser-generated Rayleigh scattered light and fluorescence background must be eliminated from the collected backscattered signal before spectral interpretation (Bocklitz et al., 2011; Vankeirsbilck et al., 2002). Process monitoring using Raman spectroscopy has not yet gained the same popularity as NIR, but this may change with; recent instrument improvements through the implementation of better lasers, new spectrometer designs, improved detectors with increased sensitivity and applying computers and software, which are able to overcome the challenge of fluorescence (Bakeev, 2005).

Altogether, Raman spectroscopy is a promising monitoring technology, with various configurations capable of analyzing concentrations of substrates and products during fermentation processes (Sivakesava et al., 2001; Shaw et al., 1999; Shope et al., 1987). Quantifications with high degrees of accuracy have recently been achieved by monitoring multiple process parameters simultaneously in yeast fermentations and mammalian cell culture bioprocesses using *in situ* Raman spectroscopy and chemometrics (Abu-Absi et al., 2010; Ávila et al., 2012; Gray et al., 2013; Li et al., 2013; Uysal et al., 2013; Whelan et al., 2012). Chemometrics combines spectroscopy with statistical methods and most often includes multivariate data analysis, which enables quantitative analysis of complex data with multiple independent variables for each dependent variable. It has been used extensively with NIR where absorption bands tend to overlap (Roggo et al., 2007; Wold et al., 2001). Raman spectra with its narrower peaks should in some cases enable sufficient quantification using simple univariate calibration models, thus reducing the need for complicated chemometrics. Quantification of concentrations by applying Raman data with single or multi-linear regression is reasonable, since the intensity of Raman scattered light is directly proportional to the number of molecules (Pelletier, 2003). This linear correlation between concentration and Raman intensity can; however, deviate by resonance Raman scattering if the excitation wavelength is near an electronic absorption band (Liu and Berg, 2012). The linear relationship between Raman intensity and concentration can also be disturbed by the presence of microbial cells or signal-attenuating particles due to diffuse Lorenz–Mie scattering responsible for optical density (Butler, 1964). For a long time the challenge of removing the fluorescence background signal was considered a major obstacle in working with Raman spectroscopy. However, with better software and algorithms to deal with this; the next important issue to solve may be the development of ways to overcome the effects of light scattering particulates.

The relationship between light extinction and the concentrations of light-scattering particulates can be expressed by a simple linear correlation in dilute suspensions. In more turbid media, attenuations of Raman signals are more mathematically complicated to quantify and correct, because of multiple scattering of photons and shadow effects. Instead, the overall magnitude of Mie scattering can be revealed by using a component unchanged through the reaction and with peaks not shared by other components in the reaction mixture as an internal standard. The changes in intensity of the internal standard can only be explained by changes in scattering, if the concentration remains constant; which can be used to estimate the reductions of all other Raman signals through a relative simple scattering correction method (Aarnoutse and Westerhuis, 2005). This method of correcting for signal extinction using an attenuation coefficient was demonstrated by using sulfate in the media as an internal standard when monitoring yeast fermentation (Picard et al., 2007).

The amount of light-scattering particulates is limited in fermentations with defined media compared to lignocellulosic bioethanol production processes, where large amounts of biomass debris must be anticipated. Therefore, monitoring highly heterogeneous lignocellulosic biofuels production processes with Raman spectroscopy is complicated by the attenuation of the Raman signals due to particulates. There have been a number of publications investigating biomass structures or spectral features from components in lignocellulosic biomass using Raman spectroscopy (Stewart et al., 1995; Adapa et al., 2011; Agarwal et al., 2012; Barsberg et al., 2006; Xu et al., 2013). Until recently, minimal research has been done on how to quantify component concentrations during biorefinery processes using Raman spectroscopy. Glucose and xylose have been measured off-line simultaneously in samples taken from corn stover hydrolysate applying extraction procedures to improve detection (Shih et al., 2011). In another recently published study, HPLC data of ethanol and glucose concentrations obtained from a lignocellulosic fermentation of switch grass hydrolysate correlated well with real-time Raman derived results (Ewanick et al., 2013). These two studies were based on monitoring fermentation of the separated liquid fractions or filtered samples without interference from light scattering biomass debris. In future large scale biorefineries, continuous removal of all scattering solids in the production processes is not a realistic scenario and some degree of particulates should be expected and accounted for in the method used for real-time monitoring.

The purpose of our research was to examine the application of Raman spectroscopy in monitoring conversion processes involved in the production of lignocellulosic bioethanol, considering disturbance from particulates. Experiments were conducted with the aim of developing methods for real-time quantification of substrate, product, and inhibitor concentrations during dilute acid pretreatment, enzymatic hydrolysis, and fermentation with *Saccharomyces cerevisiae*. The microcrystalline cellulose Avicel was used as a model substrate for the initial hydrolysis experiment. The analysis of the model substrate was followed by the analysis of more complicated lignocellulosic material by monitoring pretreatment, hydrolysis, and fermentation of sugarcane bagasse. Applying complicated multiple linear regression or partial least squares analysis was avoided; and quantification was carried out with only simple linear regression with one independent variable after initial correction of attenuated Raman intensities. We demonstrate real-time monitoring of a *S.cerevisiae* fermentation process using a Raman instrument equipped with a sapphire ball probe designed to minimize interference by particulate as well as withstand the harsh environment during sterilization. We examined the prospect of using Raman spectroscopy for monitoring concentration changes during the pretreatment, hydrolysis, and fermentation processes. Scattering correction of the spectra by using an internal standard in the media was performed before quantification of concentrations.

2. Methods

2.1. Avicel hydrolysis and fermentation

A one liter suspension of 40 g/L Avicel® PH-101 (~50 μm particle size) microcrystalline cellulose (Sigma–Aldrich, USA), 20 g/L proteose peptone, and 10 g/L yeast extract, was adjusted to pH 5.5. This suspension was hydrolyzed by adding 10 mL (0.36 mL product/g cellulose) of Accelerase® 1500 (DuPont, USA) at 50 °C and 100 rpm for 48 h in a 1.4 L Multifors bioreactor (Infors-HT,