Bioresource Technology 102 (2011) 9675–9682

Contents lists available at ScienceDirect

Bioresource Technology

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

Modelling real-time simultaneous saccharification and fermentation of lignocellulosic biomass and organic acid accumulation using dielectric spectroscopy

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ARTICLE INFO

Article history:
Received 28 April 2011
Received in revised form 15 July 2011
Accepted 20 July 2011
Available online 27 July 2011

Keywords:
Lignocellulose
Perennial ryegrass
Biomass probe
Enzyme hydrolysis
Fibre

ABSTRACT

Dielectric spectroscopy (DS) is routinely used in yeast and mammalian fermentations to quantitatively monitor viable biomass through the inherent capacitance of live cells; however, the use of DS to monitor the enzymatic break down of lignocellulosic biomass has not been reported. The aim of the current study was to examine the application of DS in monitoring the enzymatic saccharification of high sugar perennial ryegrass (HS-PRG) fibre and to relate the data to changes in chemical composition. DS was capable of both monitoring the on-line decrease in PRG fibre capacitance (C = 580 kHz) during enzymatic hydrolysis, together with the subsequent increase in conductivity (G = 580 kHz) resulting from the production of organic acids during microbial growth. Analysis of the fibre fractions revealed >50% of HS-PRG lignocellulose had undergone enzymatic hydrolysis. These data demonstrated the utility of DS biomass probes for on-line monitoring of simultaneous saccharification and fermentation (SSF).

1. Introduction

Lignocellulosic biomass offers the potential, following saccharification, of providing a sugar rich feedstock for microbial bio-conversion into liquid transport fuels (ethanol, butanol) or high-value platform chemicals. Separate hydrolysis and fermentation (SHF) can be used in the production of microbial derived fuels and chemicals from biomass, however continued interest has grown around simultaneous saccharification and fermentation (SSF). SSF is a process whereby both the release of sugars from biomass and subsequent bioconversion occur in the same vessel at the same time, which has the combined benefit of reducing both running costs and enzyme feedback inhibition during the saccharification process (Olofsson et al., 2008). Optically pure lactic acid, a commercially important organic acid, can be biologically produced from microbial fermentations for use in pharmaceutical and food industries and, following polymerisation, forms a thermoplastic polyester for use as a bio-degradable alternative to petro-chemical derived plastics (Drumright et al., 2000). Many feedstocks such as wheat straw, sugarcane bagasse and corn stover are recalcitrant to enzymatic digestion as a result of physicochemical, structural and compositional factors and require physical and/or chemical pre-treatment prior to hydrolysis (Alvira et al., 2010).

1.1. High sugar perennial ryegrass (HS-PRG)

Grasses, in particular ryegrasses, offer an alternative feedstock for the production of biofuels and bulk chemicals. These grasses are abundant, with permanent grasslands accounting for 58.5 million hectares (29%) of the total utilised agricultural area in the EU-27 countries (European Commission Eurostat, 2007). For the UK, the proportion of agricultural land set to grass was even greater, with 7 million hectares (38%) as permanent pasture and a further 6.5 million hectares (35%) designated as rough grazing (DEFRA, 2006). Genetic improvement of the dry matter digestibility (DMD) of high sugar perennial ryegrass (HS-PRG) in temperate regions has been a focus of plant breeding efforts within the UK, with DMD gains being achieved of 10 g kg^{-1} per decade, resulting in high water soluble carbohydrate (WSC > 25%) content, and, compared to woody biomass crops, low levels of both lignin (<5%) and cellulose crystallinity (33–38.6%) (Allison et al., 2009; Fahmi et al., 2007; Fahmi et al., 2008; Liu et al., 2006; Wilkins, 1997; Wilkins and Humphreys, 2003). The level of lignin and/or cellulose crystallinity

Abbreviations: DS, dielectric spectroscopy; HS-PRG, high sugar perennial rye grass; SHF, separate hydrolysis and fermentation; SSF, simultaneous saccharification and fermentation; HPLC, high performance liquid chromatography.

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doi:10.1016/j.biortech.2011.07.084

have been shown to negatively influence the ability of cellulases to hydrolyse cellulose to glucose, with lignocellulose requiring pretreatment to open the biomatrix for enzyme accessibility (Möller, 2006; Pederson and Meyer, 2010). Primarily improved for livestock production, perennial ryegrass now represents a premium feedstock for bio-refining both in terms of its high water soluble carbohydrate content and cell-wall characteristics (digestibility).

1.2. Dielectric spectroscopy (DS)

Mathematical models used to determine growth rate and substrate consumption/product formation are often/commonly dependent on biomass data; as such, cellular biomass represents an important fermentation variable (Ferreira et al., 2005; Sarra et al., 1996). By application of an electric field, dielectric spectroscopy (DS) biomass probes measure the capacitance that reflects the extent of charge separations (polarisations) induced in a suspension of living cells within the fermentation media. An increase in capacitance is correlated with the increase in viable biomass resulting from microbial/mammalian cellular growth, which can thereby enable fermentation scientists to model growth rate. Within industrial settings, capacitance probes are routinely used for on-line monitoring of microbial and mammalian cell biomass growth inside fermentation vessels (Carvell and Dowd, 2006; Ferreira et al., 2005; for review see: Harris et al., 1987; Kell et al., 1990).

1.3. Investigative goals

In previous enzymatic studies we have noted two phenomena of HS-PRG fibre digests and have sought to resolve them; (1) the presence of a thermo-tolerant microbe capable of utilising grass lignocellulose derived sugars as a carbon source during hydrolysis at 50 °C (Pippel, 2010) and (2) a decrease in capacitance (pF cm^{-1}) values recorded on-line during enzymatic saccharification of grass fibre.

While Markx et al. (1991) demonstrated the potential of DS to monitor plant cell disruption through shear sensitivity, to our knowledge there has been no systematic investigation on the use of DS for on-line monitoring of enzyme mediated lignocellulose fibre degradation, while simultaneously monitoring microbial growth during fermentation. In this paper we address this application and discuss the use of DS in the context of bioconversion of HS-PRG fibre by simultaneous saccharification and fermentation (SSF).

2. Methods

2.1. High sugar perennial grass fibre (HS-PRG)

Frozen AberMagic HS-PRG, 800 g, was thawed at room temperature, juiced using a GreenStar GS-1000 juice extractor and the fibre stored at −18 °C until required. The moisture content of the de-juiced grass fibre was determined by re-weighing a known quantity of fibre following 16 h drying at 80 °C. In triplicate, 117.4 g wet weight of fibre, ground to a fine powder in liquid nitrogen, was added to an Infors-HT labfors 1L water jacketed bioreactor without baffles, the volume adjusted to 5% dry matter (DM) w/v with deionised water and, following pasteurisation at 70 °C, the requisite enzyme cocktail added. The pH, temperature and impeller (twin Rushton 54 mm × 12 mm) speed were adjusted to pH 5.5, 50 °C and 500 rpm respectively with constant monitoring and these conditions were maintained throughout the saccharification and fermentation process using Iris 5.0 software.

2.2. Enzymatic saccharification of grass fibre

All enzymes were kindly supplied by Biocatalysts Ltd., Cefn Coed, Parc Nantgarw, CF15 7QQ, UK. Cellulase™ 13L, endo-xylanase Depol™ 761P and pentosanase Depol™ 453P were applied in accordance with the manufacturer's recommendations at 5%, 1.5% and 0.025% w/w DM, corresponding to 75, 13.2 and 1.3 units g DM^{-1} respectively. Ferulic acid esterase, Depol™ 740L was added at 0.25% w/w DM, corresponding to 0.09 units g DM^{-1}. One unit of activity was defined as the amount of enzyme required to liberate I unit of substrate per minute.

Sterile enzyme saccharifications of HS-PRG fibre, contained 50 μg mL^{-1} chloramphenicol, with active (BR-1) and denatured (BR-2) enzyme cocktail. Enzymes were denatured by heating for 1 h at 100 °C. SSFs were conducted in duplicate bioreactors (BR-A and BR-B) containing active enzyme. One bioreactor (BR-C) containing denatured enzyme cocktail heated for 16 h at 100 °C was used as a control. When all bioreactors were equilibrated to pH 5.5 at 50 °C, the optimal conditions specified by the manufacturer, saccharification was initiated by adding the requisite enzyme cocktail.

2.3. Reducing sugar analysis and chromatography

Two-millilitre samples were removed from each bioreactor at various time intervals using a 20 mL syringe and cannula stored in 70% ethanol and rinsed in sterile water just prior to sampling. Following centrifugation, 21,000*g* for 5 min at room temperature, samples were diluted 1 in 5 with H_2O and assayed for reducing sugars (Miller, 1959). Briefly, 250 μL of diluted sample was added to 250 μL reducing sugar reagent containing 10 mg mL^{-1} dinitrosalicylic acid, 2 mg mL^{-1} phenol, 500 mg mL^{-1} sodium sulfite, 10 mg mL^{-1} NaOH, mixed and heated at 90 °C for 15 min. On cooling to room temperature 100 μL of 30% potassium sodium tartrate was added, the absorbance read at 575 nm, and following subtraction of a water blank, quantified against the absorbance value of a 1 mg mL^{-1} glucose standard.

In preparation for HPLC, 50 μL aliquots were added to 950 μL 5 mM H_2SO_4 containing 5 mM crotonic acid as an internal standard and passed through a 0.45 μm filter into an autosampler vial and capped. Samples were simultaneously assayed for carbohydrates and organic acids by injecting 25 μL of sample onto a Jasco 1500 series HPLC system fitted with a Rezex ROA organic acid H^+ column 300 mm × 7.8 mm (held at 35 °C) and an RI-2031 refractive index detector. The mobile phase was 5 mM H_2SO_4 at a flow of 0.6 mL min^{-1} and the data acquired using EZChrom Elite software and quantified against a series of carbohydrate and organic acid standards consisting of sucrose, glucose, xylose, arabinose, and lactic, acetic, formic, propionic and butyric acids 2.5 mg mL^{-1} to 10 mg mL^{-1}. Oligomeric carbohydrates were quantified as sucrose equivalents and polymeric carbohydrates were quantified as fructan equivalents against isolated grass fructan, 7 mg mL^{-1} to 105 mg mL^{-1}.

2.4. Capacitance and conductivity calibration and data acquisition

Capacitance (C) and conductivity (G) measurements were taken via an Aber Instruments' (AI) BM220 biomass monitor using an AI 12 mm annular probe for each of three channels. To adjust data acquisition for the environment of each bioreactor, each probe was pre-calibrated by AI, sterilised in 70% ethanol and re-calibrated in situ utilising AI's custom probe calibration procedure. Briefly, re-calibration was performed using NaCl solutions corresponding to conductivities of 3, 14 and 30 mS cm^{-1}. Data were acquired simultaneously from each channel by frequency scanning in a range of 100 kHz to 20,000 kHz at 1 min intervals over the experimental time period, processed using the β-dispersion equation [t1];

$$C(f) = C\infty + \Delta C * (1 + (f/fc)^{(1-\alpha)} * \sin(\pi * \alpha/2))/(1 + 2 * (f/fc)^{(1-\alpha)} * \sin(\pi * \alpha/2) + (f/fc)^{(2-2*\alpha)})$$