

OVERVIEW

Switchgrass has been targeted as a potential perennial, herbaceous biomass crop to replace maize corn as a source of domestically-produced bioethanol. Current commercial bioconversion methods frequently employ enzymatic degradation of lignocellulosic feedstocks, such as switchgrass, to the simpler cellosextrins, which are further metabolized to ethanol, the end product. We are interested in characterizing the progression of the lignocellulosic bioconversion to release cellosextrins. We show that this process could be monitored using an ultrahigh pressure liquid chromatographic (UPLC) separation of the cellosextrins coupled with electrospray ionization and a 4000 QTrap hybrid triple quadrupole/linear ion-trap mass spectrometer. Cellobiose, cellobiose, cellobiose, and cellobiose are separated in less than two minutes with high reproducibility. The calculated detection limit is typically less than 50 fmol for each species.

INTRODUCTION

Ethanol produced from cellulosic biomass, the most abundant biological renewable resource, is the leading alternative to petroleum-derived transportation fuel. The initial conversion of biomass into sugars is the key obstacle in overcoming the recalcitrance of lignocellulosic biomass and ultimately lowering the cost of biofuel production. Microbial degradation of cellulosic biomass can be accomplished by a vast array of microorganisms for the primary hydrolysis of cellulose to yield soluble cellosextrins that range from two to six degrees of polymerization (DP).

Cellosextrin DP can be determined by a number of approaches, including classical osmometry, freezing point depression, and boiling point elevation, but all of these are tedious and require large amounts of sample. A sensitive, rapid and accurate assay for the resolution of specific cellosextrins could be used to establish a high throughput microbial screening platform that would discover novel cellulose-degrading microorganisms and provide valuable insights into the manner and efficiency of a given microorganism to degrade cellulosic substrates.

We describe here a new assay for cellosextrins employing an initial ultra-high pressure liquid chromatographic (UPLC) separation followed by mass spectrometric detection of sodiated cellosextrins. The assay was successfully applied to the digests of both pure cellulose and switchgrass.

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- Zhang Y-HP, Lynd LR (2006) Biosynthesis of radio-labeled cellosextrins by the *Clostridium thermocellum* cellobiose and cellobiose phosphoribosyltransferases for measurement of intracellular sugars. *Appl Microbiol Biotechnol* 70:123-129.
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Evaluation of the Cellosextrin Profiles of the Enzymatic Digests of Switchgrass

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CONCLUSIONS

Ultra-high pressure chromatography (UPLC) provides a rapid and convenient approach for characterizing the cellosextrins present in bioenergy digests.

- Sample-to-sample analysis time is 6 min.
- Four smallest cellosextrins are well-resolved.

Electrospray ionization mass spectrometry in the positive-ionization mode with single-ion monitoring mode permits detection limits less than 50 pmol for three of the four target compounds based on the sodiated molecule.

Other sugars may co-elute with a given cellosextrin. Simply matching a retention time and m/z with standard values does not permit conclusive identification.

Other eluents and modifiers may permit MS/MS-procedures that are inherently more selective and sensitive than those employing SIM.

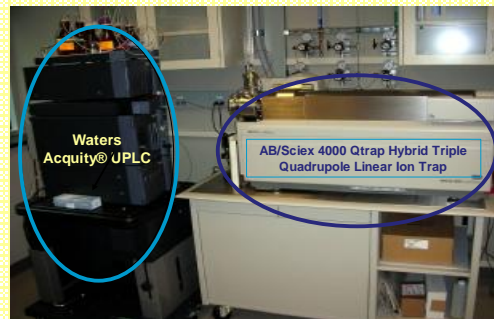
Multiple dilutions are frequently needed for proper quantitation. The current dynamic range of the method is approximately a factor of 30 – insufficient for authentic samples with a single dilution whose cellosextrin concentrations may vary by at least a factor of 50.

The organism OB47 metabolizes dried sieved switchgrass and pure cellulose at very different rates, producing dramatically different solution cellosextrin levels at similar time points in bioreactor studies.

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APPARATUS



The four smallest cellosextrins were separated using a 2.1 mm i.d. x 100 mm BEH HILIC column employing a 0.5 μ L injection volume. A gradient separation employing acetonitrile (10 μ M sodium acetate, Solvent A) and water (10 μ M sodium acetate, Solvent B) was employed, as shown below. The sample-to-sample analysis time was 6 min.

Time, min	% A (v/v)	% B (v/v)	Flow rate, mL/min
Initial	80	20	0.5
1.0	80	20	0.5
2.0	70	30	0.5
2.1	5	95	0.5
3.0	5	95	0.5
3.1	80	20	0.5

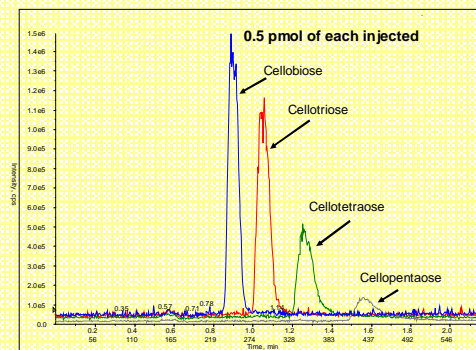
The cellosextrins were detected as their sodiated analogs, m/z 365, 527, 689, 851, for cellobiose, cellobiose, cellobiose, and cellobiose, respectively. The AB/Sciex 4000 Qtrap employed electrospray ionization in the positive ionization mode. All ions were monitored in the single-ion monitoring (SIM) mode. The instrument was optimized for detection of the four target m/z values.



All biodegradations were performed using 1-1.5% (w/v) dried sieved switchgrass or Avicel (cellulose) in a 5-L glass reactor. The organism OB474 was chosen to degrade either feedstock. The temperature and pH were maintained under servo control at 80 °C and 7, respectively. Samples were withdrawn at time points up to 260 hr. All samples were filtered through a 0.2 μ m porosity Teflon syringe filter prior to UPLC/MS analysis.

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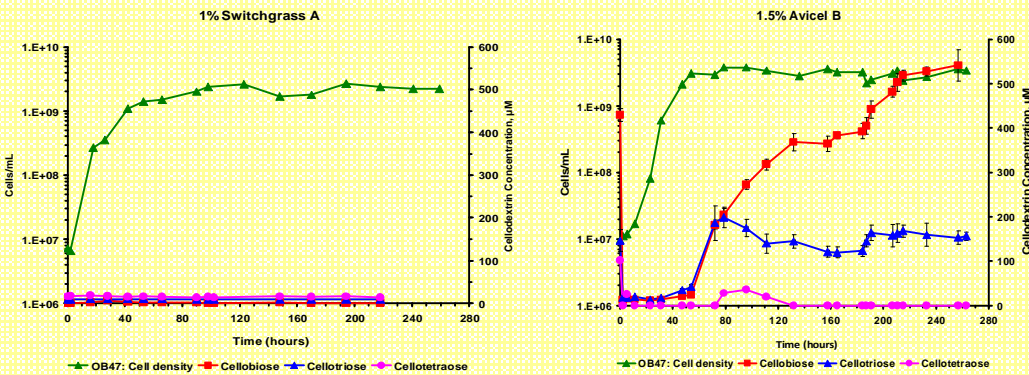
RESULTS



Compound	m/z (sodiated species)	Retention time, min (mean \pm std deviation, n = 4)
Sucrose	365.0	0.87 \pm 0.005
Cellobiose	365.0	0.89 \pm 0.005
Maltose	365.0	0.89 \pm 0.000
Trehalose	365.0	0.95 \pm 0.000
Melibiose	365.0	0.96 \pm 0.005
Maltotriose	527.1	1.03 \pm 0.006
Cellobiose	527.1	1.03 \pm 0.005
Raffinose	527.1	1.08 \pm 0.006
Maltotetraose	689.1	1.22 \pm 0.006
Cellobiose	689.1	1.24 \pm 0.008
Stachyose	689.1	1.39 \pm 0.006
Maltopentaose	851.1	1.47 \pm 0.005
Cellobiose	851.1	1.54 \pm 0.010

- Calibration curves were linear over a twenty-fold range, from 0.025-0.5 pmol injected per compound. Usable, but nonlinear, calibration curves could be obtained up to approximately 0.75 pmol injected per compound.
- The detection limits were estimated from the linear calibration curves ($3 s_{y/x}$ /slope, where $s_{y/x}$ the standard error of the y value estimates, is assumed to approximate the standard deviation of the blank, sB). The detection limits for cellobiose, cellobiose, cellobiose, and cellobiose were 39, 19, 16, and 154 fmol, respectively, based on four-fold replication.
- With time, cellobiose was not observed in either the standards or the samples and was presumed irreversibly adsorbed by, or precipitated onto, the HILIC column. Further work employed results from only cellobiose, cellobiose, and cellobiose.
- Isomeric sugars used to "start" the degradation culture may also be present. Some, but not all, of these could be resolved from the cellobiose themselves.

Comparison Between the Degradation of Switchgrass and Avicel® (pure cellulose)



- All points represent the average of triplicate injections at each timepoint.
- The same dilution (1/5) was used for all cellosextrins in switchgrass. For Avicel®, a 1/200 dilution was used for cellobiose and cellobiose, while a 1/50 dilution was used for cellobiose.
- All concentrations of cellosextrins in switchgrass were determined at or near the detection limit.
- The concentrations of cellosextrins in switchgrass were up to one-hundred fold smaller than those in Avicel®.

DISCUSSION

ANALYTICAL CHEMISTRY AND MASS SPECTROMETRY

The ACQUITY® UPLC system equipped with a 2.1 mm i.d. x 100 mm HILIC column permits rapid and reproducible separations of the smaller cellosextrins.

- The sample-to-sample analysis time is 6 min.
- The method is robust and reliable for cellobiose, cellobiose, and cellobiose.
- Cellobiose was set aside as a marker compound.
- The calculated method detection limit for the three cellobiose markers is better than 50 fmol for each compound.

The determination of cellobiose is complicated by possible adsorption by, or precipitation onto, the surface of the packing material.

The cellobiose are not necessarily resolved from other sugars with the same molecular weight. It is not possible to identify a particular cellobiose with certainty solely from its retention time and sodiated molecule m/z value.

The linear dynamic range of the calibration curves is not more than approximately a factor of 30, between 0.025 and 0.75 pmol.

In the MS/MS mode, no distinct product ions for any of the sodiated cellobiose were observed that would permit good selected-reaction monitoring (SRM) detection.

Alternative approaches that extend the dynamic range would be beneficial.

Multiple dilutions are currently needed for proper quantitation.

- The current 1/50 and 1/200 dilutions have been arbitrarily chosen and may need to be modified.
- The current approach requires that a representative number of samples be screened in order to determine a dilution factor which permits the unknown cellobiose concentrations to fall within the upper and lower bounds of the calibration curve.
- Internal standards or the method of standard additions may be necessary for more accurate quantitation.

Large dilution factors exaggerate minor errors in peak area measurements. Background subtraction alone may be insufficient to produce reliable results.

BIOLOGY

The organism OB47 metabolizes switchgrass and pure cellulose at very different rates, producing dramatically different solution cellobiose levels at similar time points in bioreactor studies.

The nutrient buffer employed in these studies may not be optimal for switchgrass, though it may be quite satisfactory for pure cellulose.

Additional studies will be needed to determine both the optimal organism and its corresponding nutrient medium for more complete switchgrass degradation.