Development of an ASTM standard for the measurement of “cellulose”

David Mangan
dmangan@neogen.com

Overview

1) Megazyme/Neogen and the ASTM process
2) The need for a method
3) NREL assay and associated biases
4) Proposed methods and assay performance
5) Next steps
6) Conclusion

Megazyme and Polysaccharide Assay Expertise

1988
Co-founded by Prof. Barry McCleary
Megazyme Foundation

1995
β-Glucan (AOAC 995.16)

1996
Total Starch (AOAC 996.11)

1999
Fructans in food (AOAC 999.03)

2002
Resistant Starch (AOAC 2002.02)

2009-2011
First AOAC methods for dietary fiber measurement matching CODEX definition (AOAC 2009.01 & 2011.25)

2018
Fructans in animal feed and pet food (AOAC 2018.07)

2021
Neogen
Neogen Acquires Megazyme

2017-2022
Improved methods for CODEX total dietary fiber that also allow insoluble/soluble fiber split (AOAC 2017.16 & 2022.01)

Development Of An Assay For “Cellulose” Within ASTM

2021
E48.05
Dec meeting
Reintroduction of cellulosic content method discussion

E48.05
June meeting
2022
Starch methodology presentation and cellulose working group formation

Working group meets virtually six times

2022
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Agreement to move Neogen method to ballot

2023
July-Aug
E48.05
Sub-committee ballot

Sep-Oct
E48.05 Sub-,
E48 Main
committee ballot
2023

FELC 2023
FUEL ETHANOL LABORATORY CONFERENCE

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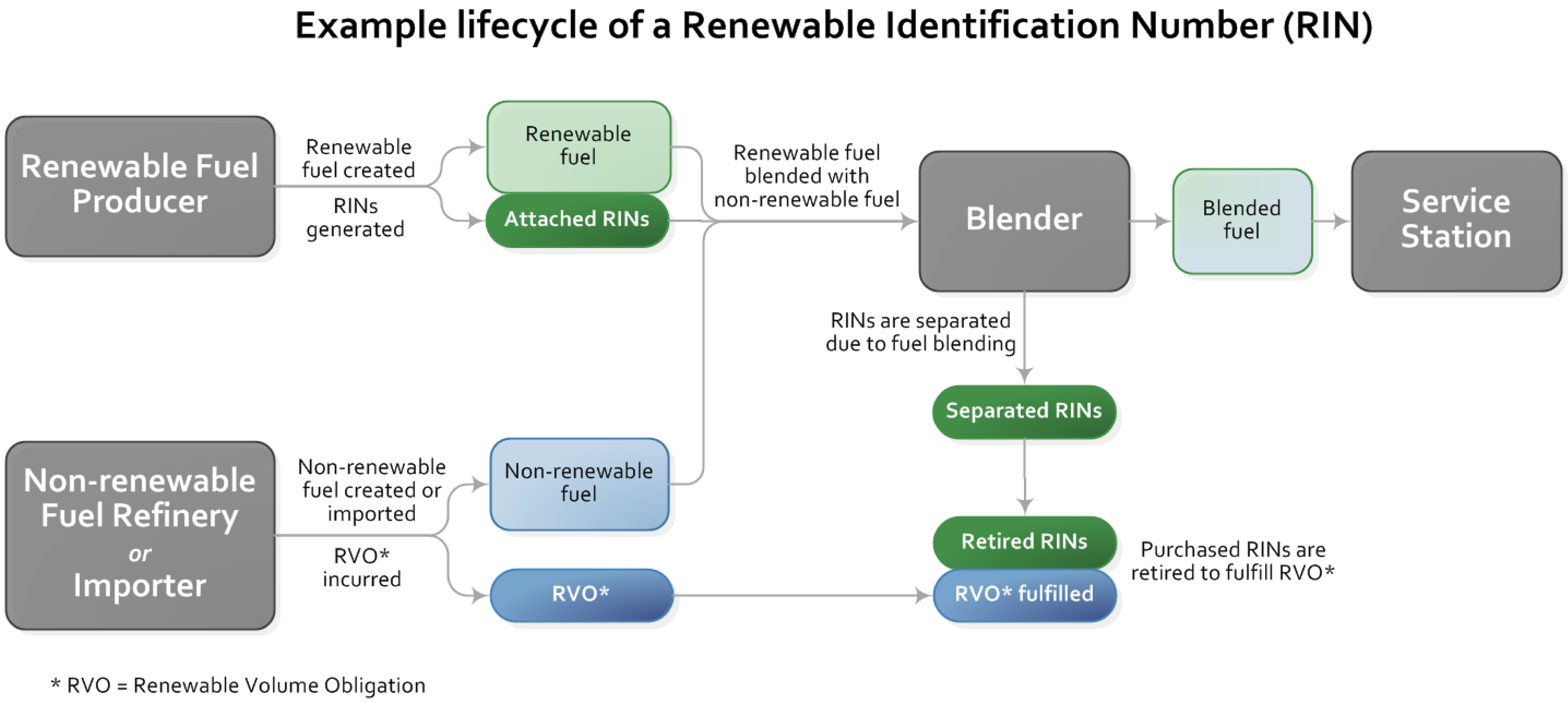
The Need For A Method

The Need For A Method



https://www.epa.gov/sites/default/files/2015-08/example_lifecycle_of_a_rin_0.png

The Need For A Method

Ethanol PRODUCER MAGAZINE

Fiber Frustration

MAY 10, 2021 BY LISA GIBSON

The stalled U.S. EPA approval of corn kernel fiber-to-ethanol pathways could be having a $1 billion impact on a 15-billion-gallon-per-year ethanol industry, says Jim Ramm, director of engineering for EcoEngineers. That's assuming 3% of overall production could be from fiber and a $2 premium. It's worst-case scenario, yes, but it's realistic, nonetheless.

eco engineers

Daily RIN, LCFS & CFP Update

D-Code	Average Price		
	2022	2023	2024
D3	$3.225	$3.220	$2.833
D4	$0.855	$0.831	$0.840
D5	$0.845	$0.825	$0.820
D6	$0.830	$0.836	$0.830

The Need For A Method

Designation: E3181 – 20

Standard Practice for
Determination of the Converted Fraction of Starch and Cellulosic Content From a Fuel Ethanol Production Facility

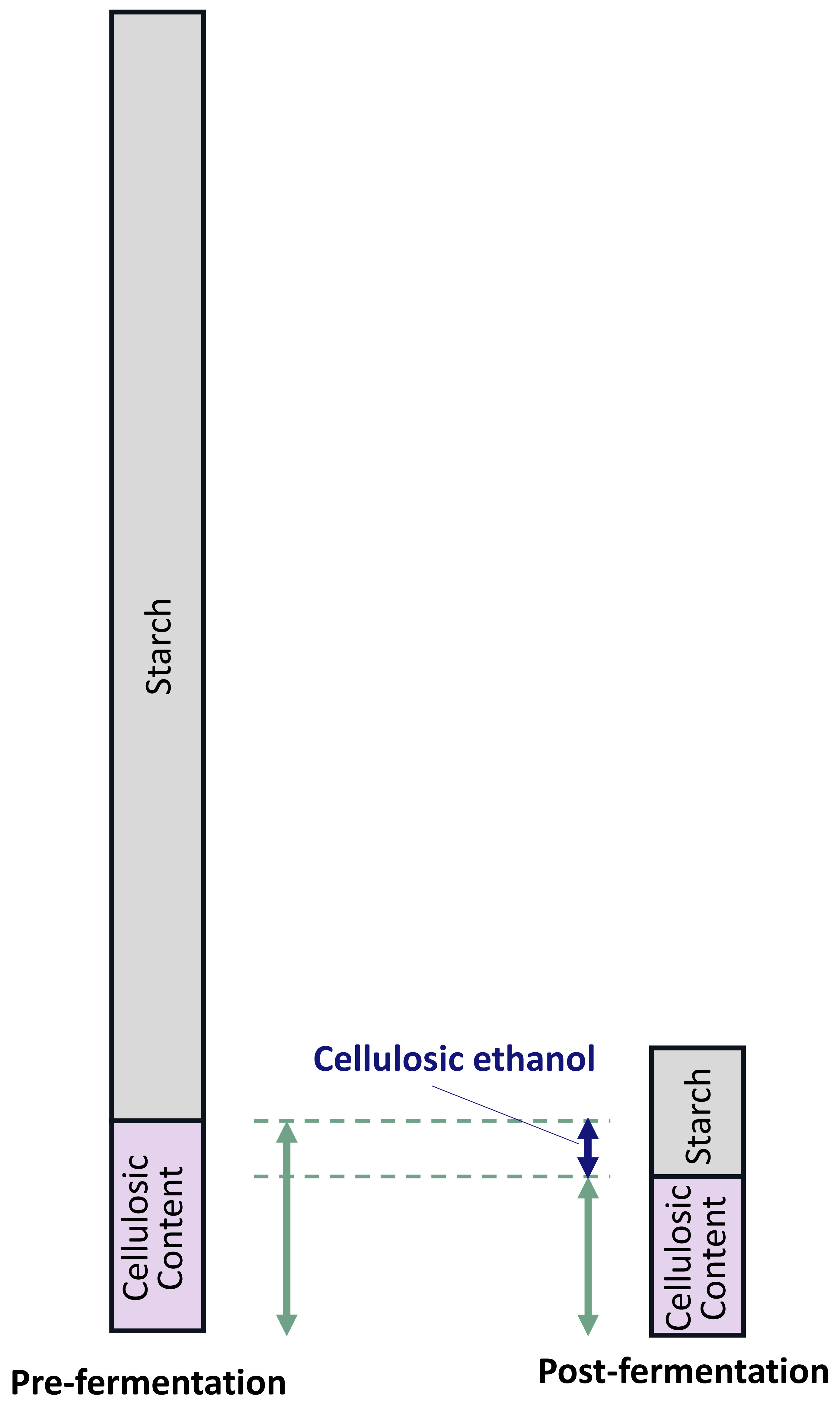
$$Ash_{ratio} = (Ash_{AC} / Ash_{BC}) \qquad (X5.3)$$

$$CF_c = 1 - ((Cellulosic\ \ Content_{AC} / Ash_{ratio}) / Cellulosic\ \ Content_{BC})$$

Where:

CF_C = cellulosic converted fraction
$_{AC}$ = After conversion
$_{BC}$ = Before conversion

Note:
EPA has defined "cellulosic content" as the sum of **cellulose, hemicellulose and lignin**



NREL Assay and Associated Biases

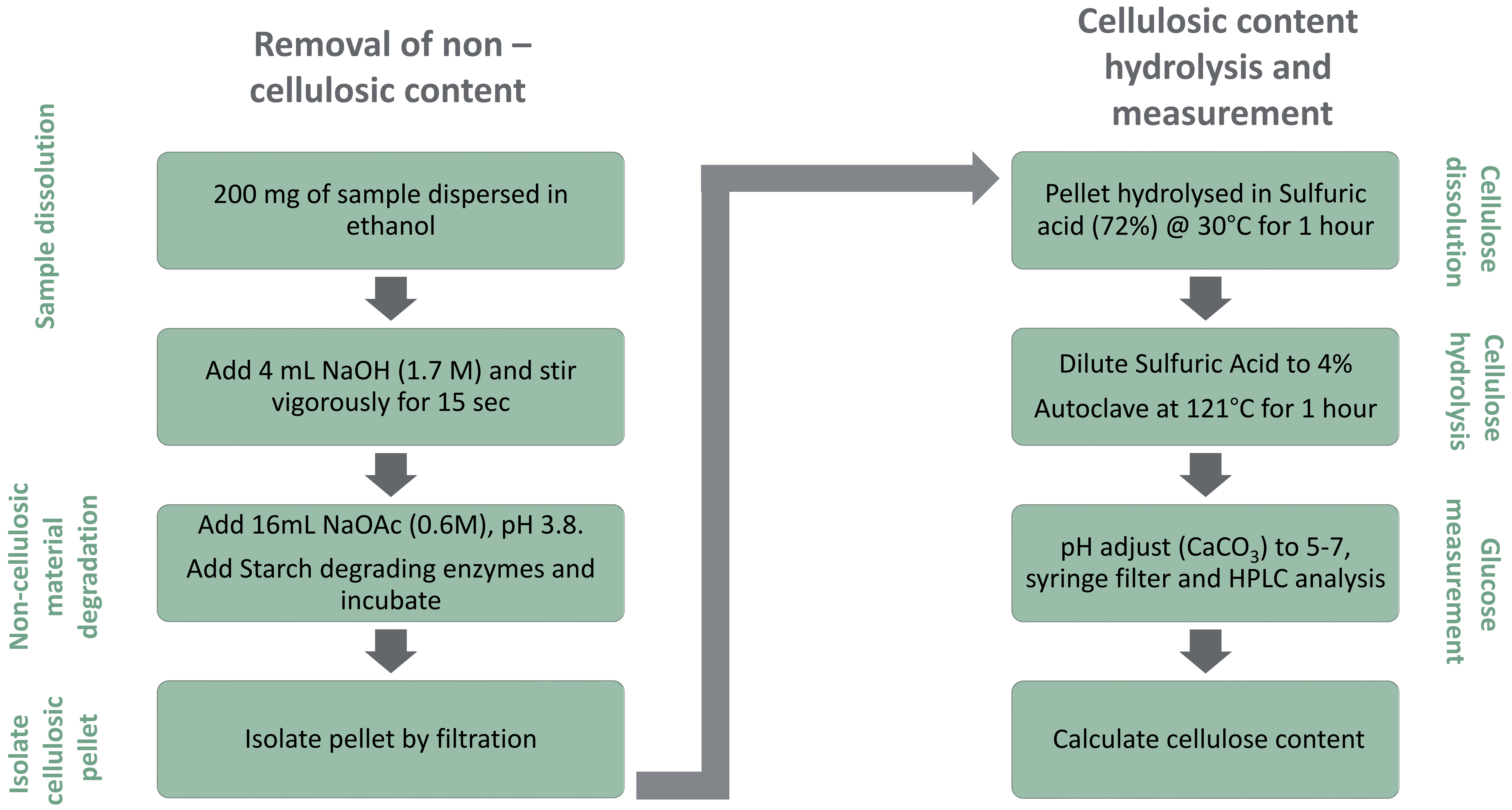
NREL assay (2021)

Cellulose (2021) 28:1989–2002
https://doi.org/10.1007/s10570-020-03652-2

ORIGINAL RESEARCH

Direct determination of cellulosic glucan content in starch-containing samples

Justin B. Sluiter · Katie P. Michel · Bennett Addison · Yining Zeng · William Michener · Alexander L. Paterson · Frédéric A. Perras · Edward J. Wolfrum



Neogen (Modified NREL) Assay (2023)

Removal of non – cellulosic content

Sample dissolution

- 200 mg of sample dispersed in ethanol
- Add 4 mL NaOH (1.7 M) and stir vigorously for 15 sec

Non-cellulosic material degradation

- Add 16mL NaOAc (0.6M), pH 3.8.
  Add Starch and **yeast carbohydrate degrading enzymes** and incubate

Isolate cellulosic pellet

- **Add ethanol to precipitate soluble cellulosic material** and isolate by filtration

Cellulosic content hydrolysis and measurement

Cellulose dissolution

- Pellet hydrolysed in Sulfuric acid (72%) @ 30°C for 1 hour

Cellulose hydrolysis

- Dilute Sulfuric Acid to 4%
  Autoclave at 121°C for 1 hour

Glucose and Galactose measurement

- pH adjust ($CaCO_3$) to 5-7, syringe filter and HPLC analysis
- Calculate cellulose/hemicellulose-derived glucan and galactan (CHDGG)

CHDGG = Cellulose/Hemicellulose-Derived Glucan and Galactan

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Bias 1 – Effect Of Yeast In Pre- And Post-fermentation Samples

Cellulose (2021) 28:1989–2002
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"We recognize that this method cannot differentiate between β-(1,4) glucans and other β-glucans present. In particular, the measurement of cellulose β-glucans in post-fermentation material will be biased high due to …fermentation yeast. An attempt to quantify β-(1,3) glucans present in the post fermentation (DDGS) sample using commercially available enzymatic assay (Megazyme K-EBHLG) … showed significant cross-activity…Until further research can be performed in this area, we are unable to address this bias."

ASTM INTERNATIONAL

Designation: E3181 – 20

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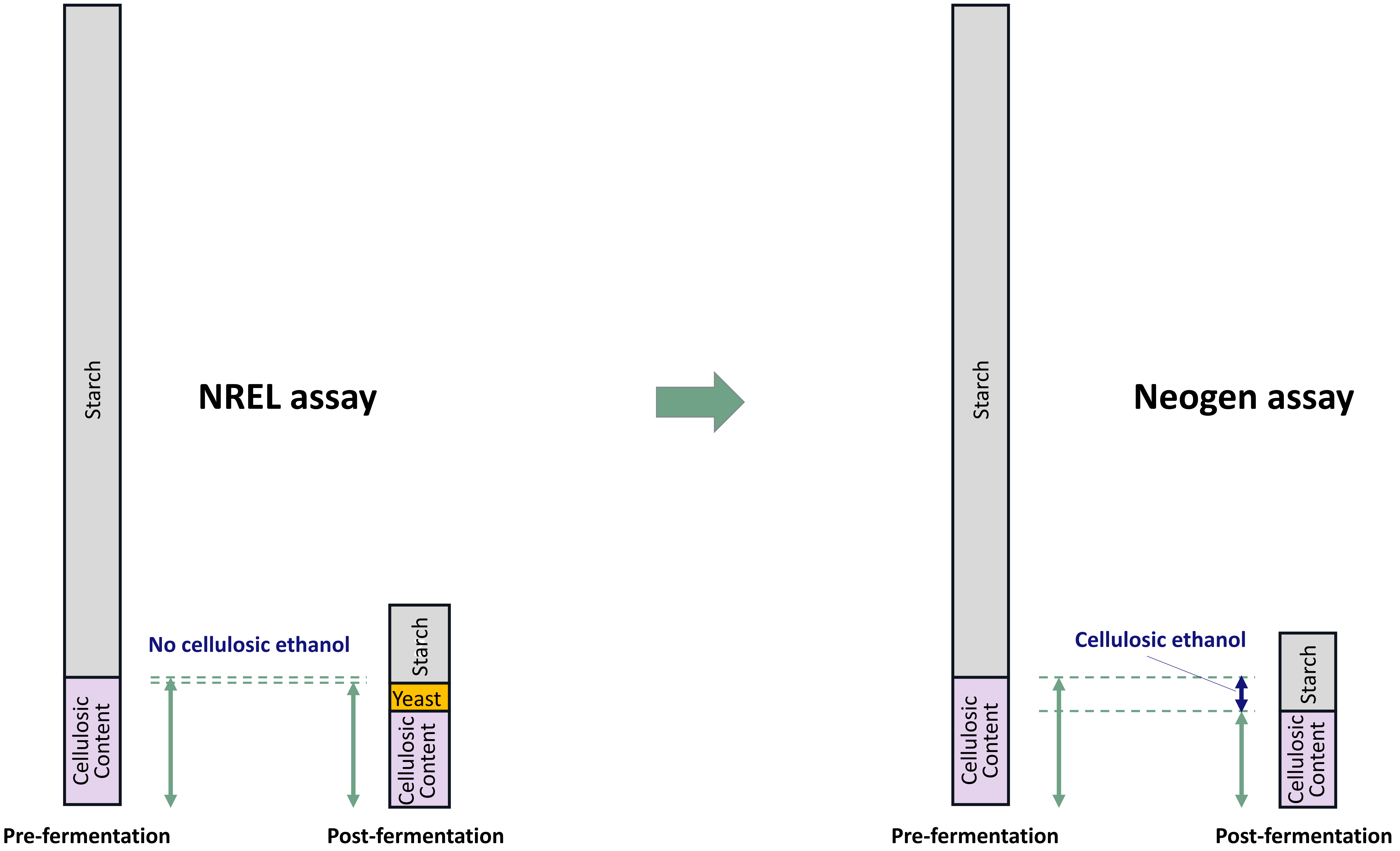
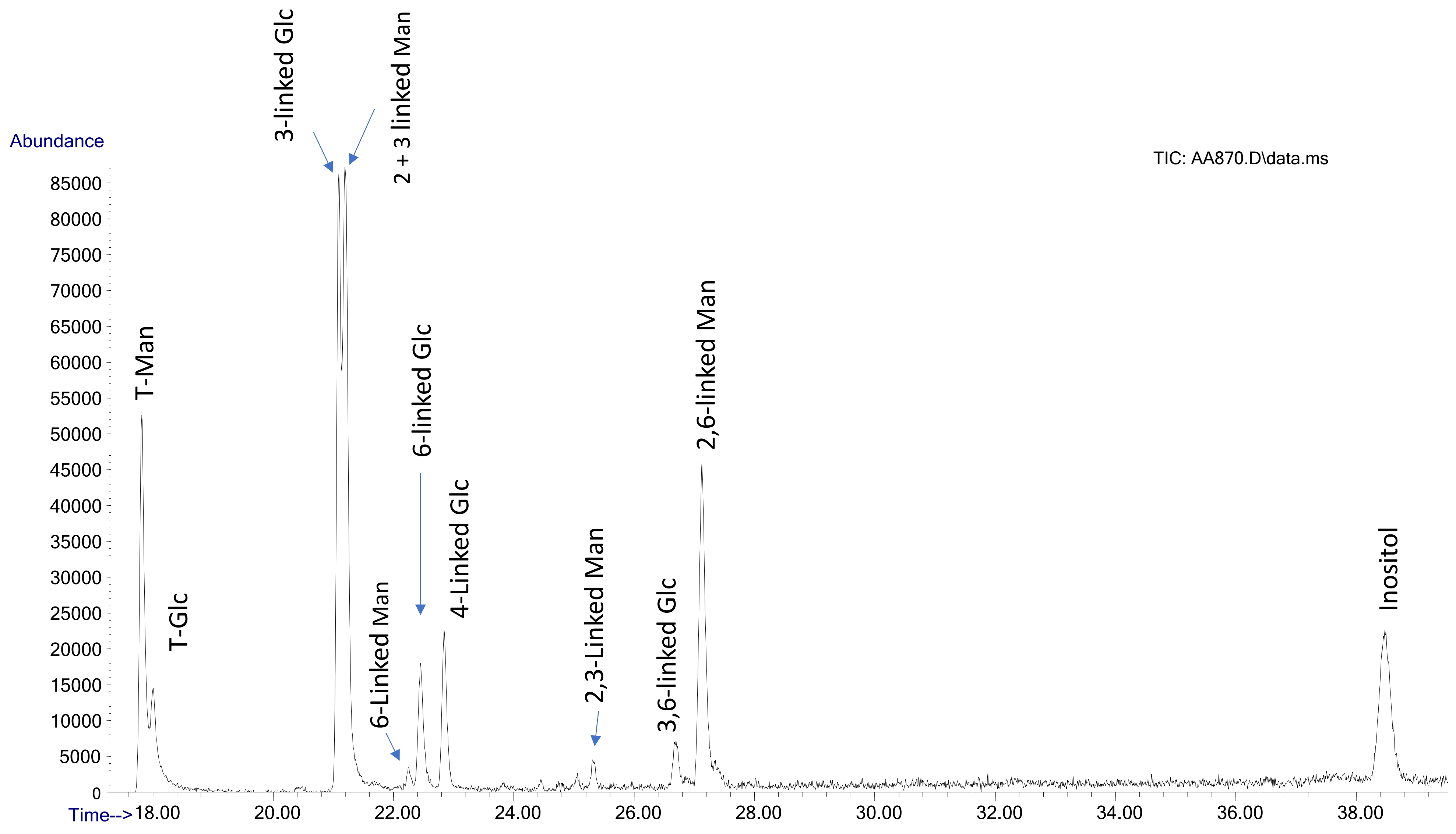
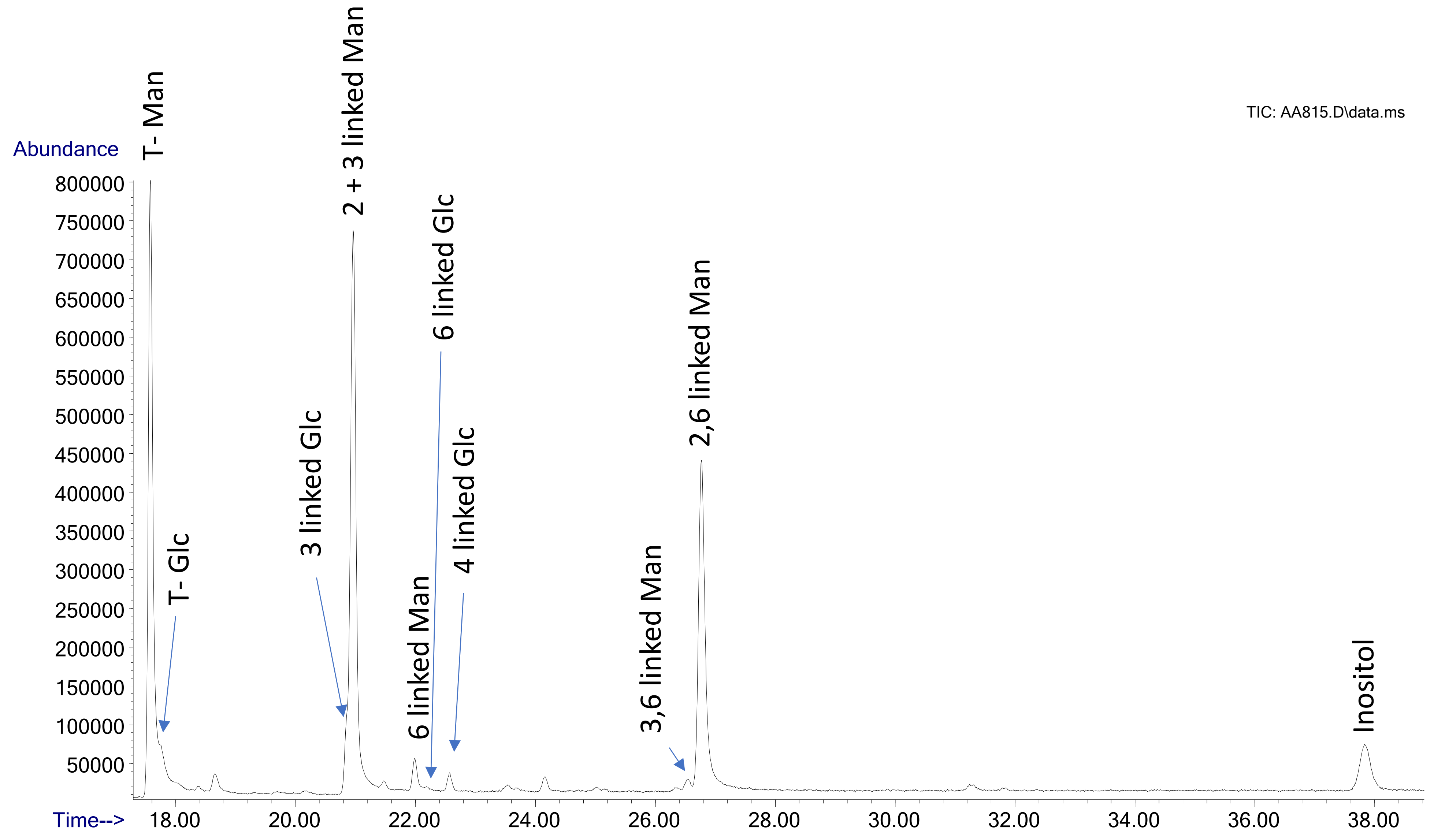
Bias 1 – Effect Of Yeast In Pre- And Post-fermentation Samples



Bias 1 – Effect Of Yeast In Pre- And Post-fermentation Samples





Sample Description	Treatment	Cellulosic glucan % (w/w) n=2	%CV
Biomass BC	None	1.86	1.59
	+ YDC	1.95	6.80
Biomass AC	None	5.73	4.42
	+ YDC	4.32	2.47

Bias 2 – Loss Of Cellulosic Content Due To NaOH Treatment

Cellulose (2021) 28:1989–2002
https://doi.org/10.1007/s10570-020-03652-2

ORIGINAL RESEARCH

Direct determination of cellulosic glucan content in starch-containing samples

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"Cellobiose, the β-(1,4)-linked glucan dimer, was chosen as the smallest molecule that retains the linkage indicative of cellulose solubilization; any detectable cellobiose would indicate cellulose loss during enzymatic hydrolysis…we conclude that there is no detectable cellobiose present in the filtrate solutions."

ELSEVIER
Carbohydrate Polymers
Volume 51, Issue 3, 15 February 2003, Pages 281-300

Degradation of cellulose under alkaline conditions

Charles J Knill, John F Kennedy

Thermal Gelation of Cellulose in a NaOH/Thiourea Aqueous Solution

Lihui Weng, Lina Zhang, Dong Ruan, Lianghe Shi, and Jian Xu

View Author Information

Cite this: *Langmuir* 2004, 20, 6, 2086–2093
Publication Date: February 18, 2004
https://doi.org/10.1021/la035995o

ELSEVIER
Bioresource Technology
Volume 251, March 2018, Pages 1-6

Cellulase pretreatment for enhancing cold caustic extraction-based separation of hemicelluloses and cellulose from cellulosic fibers

Jianguo Li [a b c], Shaokai Zhang [a c], Hailong Li [c d], Xinhua Ouyang [a], Liulian Huang [a], Yonghao Ni [a c], Lihui Chen [a]

ELSEVIER
Carbohydrate Polymers
Volume 224, 15 November 2019, 115152

Gelation of cellulose-NaOH solutions in the presence of cellulose fibers

Oona Korhonen [a], Tatiana Budtova [a b]

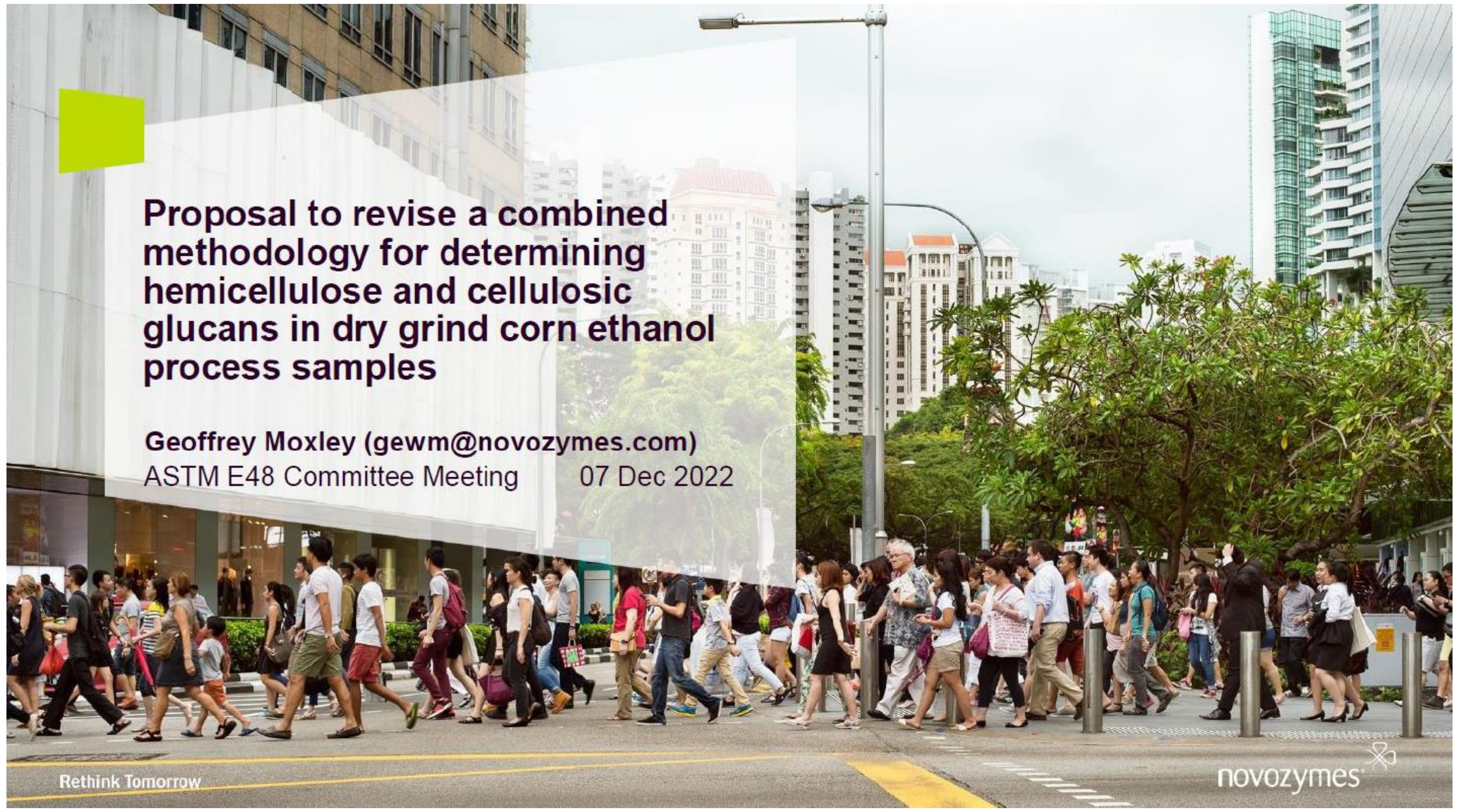
Review Paper | Published: 05 November 2015

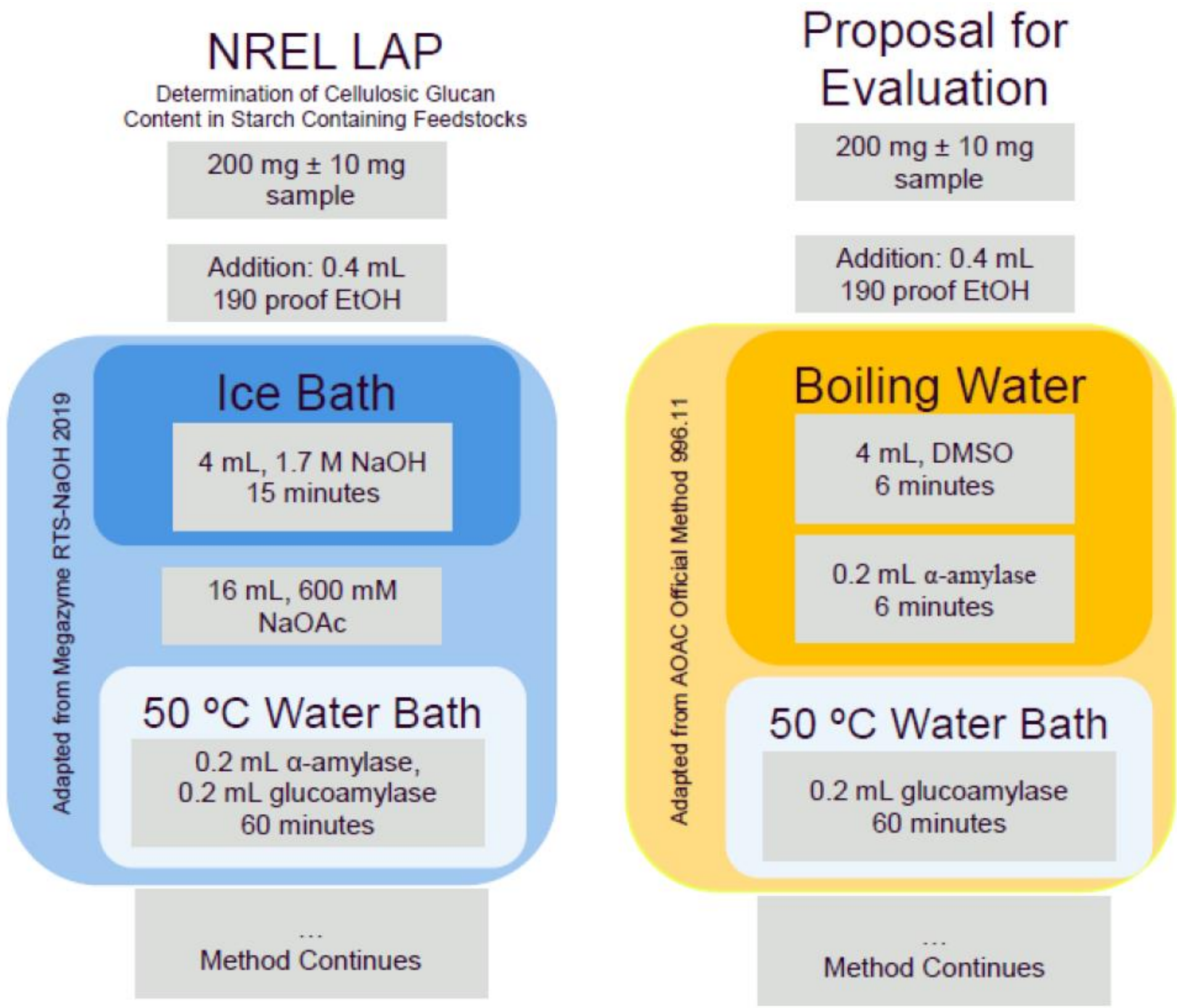
Cellulose in NaOH–water based solvents: a review

Tatiana Budtova & Patrick Navard

Cellulose 23, 5–55 (2016) | Cite this article

Bias 2 – Loss Of Cellulosic Content Due To NaOH Treatment





Bias 2 – Loss Of Cellulosic Content Due To NaOH Treatment

Sample	n, replicates	% CV	Minus (-) or Plus (+) Ethanolic Precipitation	CHDGG % (w/w)	Recaptured CHDGG % (w/w)
Conventional BC	2	1.5	-	1.69	0.43
	4	2.4	+	2.12	
Conventional AC	2	1.7	-	6.32	0.91
	4	0.3	+	7.23	
CKF Process BC	2	1.3	-	1.82	0.43
	4	0.9	+	2.25	
CKF Process AC	2	0.6	-	4.94	0.79
	4	1.1	+	5.73	

Effect of ethanolic precipitation on the recovery of cellulose in the Neogen assay

	Recovery of CHDGG versus DFO method (%)	
Sample	Without ethanolic precipitation	With ethanolic precipitation
Conventional BC	79.6	95.7
Conventional AC	80.5	102.1
CKF Process BC	88.4	95.8
CKF Process AC	74.4	99.9

Effect of ethanolic precipitation on the recovery of cellulose in the Neogen assay compared to that recovered in the DFO assay

Bias 3 – Exclusion Of Galactose In The Analyte Determined

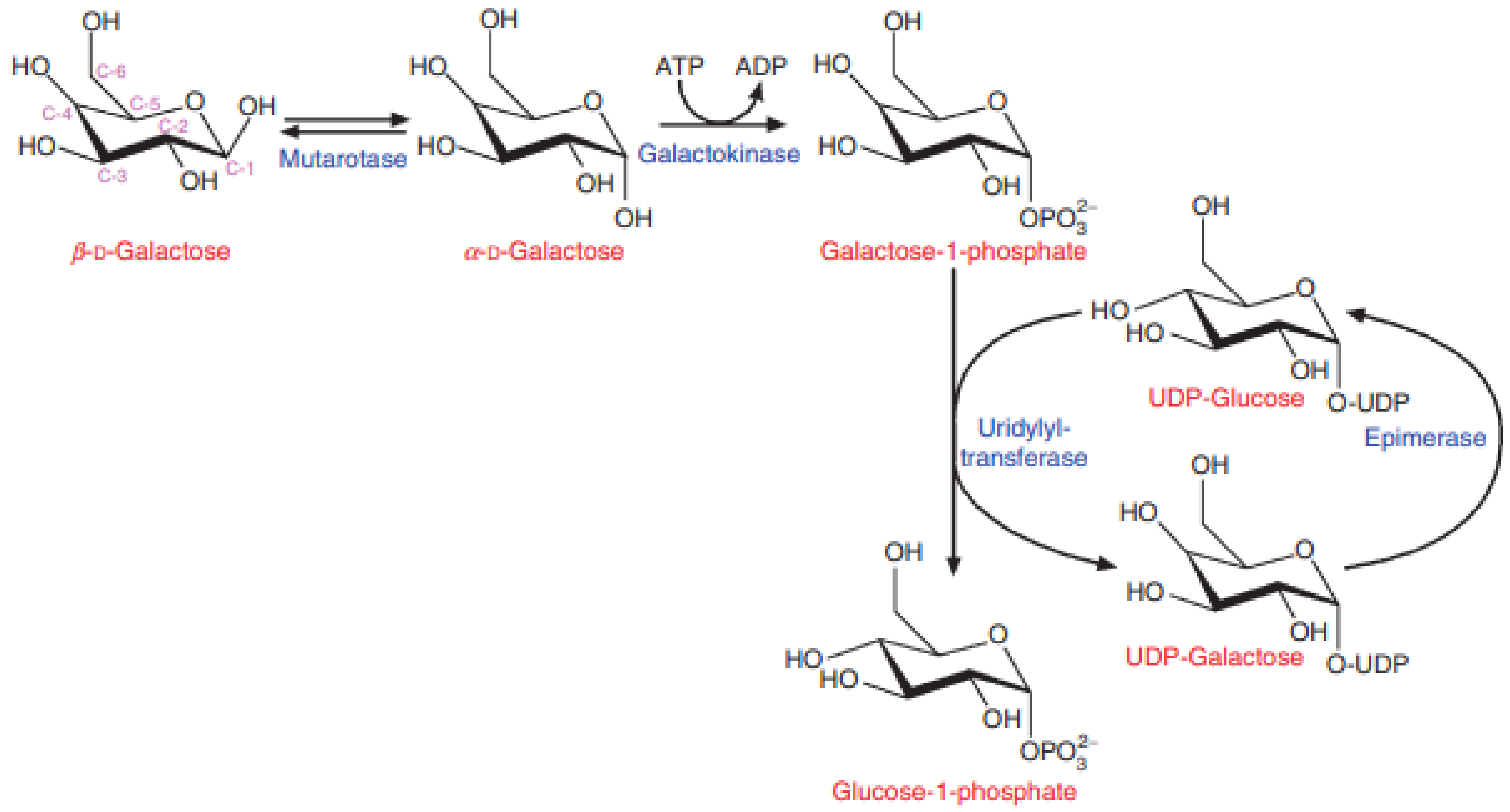


Figure 3.1 The Leloir pathway. The enzymes of this pathway promote the conversion of β-D-galactose into glucose-1-phosphate which can subsequently be used in glycolysis.

ELSEVIER
International Review of Cell and Molecular Biology
Volume 269, 2008, Pages 111-150

Chapter 3 Galactose Metabolism in Yeast—Structure and Regulation of the Leloir Pathway Enzymes and the Genes Encoding Them

Christopher A. Sellick, Robert N. Campbell, Richard J. Reece

BIOTECHNOLOGY and BIOENGINEERING

Article

Physiological studies in aerobic batch cultivations of *Saccharomyces cerevisiae* strains harboring the *MEL1* gene

Simon Ostergaard, Christophe Roca, Birgitte Rønnow, Jens Nielsen, Lisbeth Olsson

First published: 31 March 2000 |
https://doi.org/10.1002/(SICI)1097-0290(20000505)68:3<252::AID-BIT3>3.0.CO;2-K | Citations: 45

Bias 3 – Exclusion Of Galactose In The Analyte Determined

Sample Description	n	%CV	CHDGG % (w/w) DWB: Glucan only	CHDGG % (w/w) DWB: Glucan and galactan	% Cellulosic ethanol: Glucan only	% Cellulosic ethanol: Glucan and galactan
Conventional BC	4	2.44	2.21	2.64	-0.01	0.06
Conventional AC	4	0.26	7.63	8.94		
CKF Process BC	4	0.87	2.33	2.79	0.93	1.09
CKF Process AC	4	1.14	6.05	7.25		

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Proposed Methods and Performance

Comparison Of Available Open-source Methods

	NREL Method[a]	DFO method[b]	Neogen method[c]	Neogen method[d]
Sample	**% Cellulosic ethanol**			
Conventional	**-0.64**	**-0.30**	**0.11**	**0.08**
CKF Process	**0.11**	**0.22**	**1.21**	**1.01**

[a]Data from Neogen only

[b]Combined data from Neogen, POET, NREL, Novozymes

[c]Combined data from Neogen, POET, NREL

[d]Combined data from Neogen, POET, NREL - Galactan component excluded

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Investigation Into Within And Between Lab Variability

Sample	Lab	n	CHDGG % (w/w)	RSD_r	CHDGG % (w/w)	RSD_R
Conventional BC	1	13	2.76	2.86	2.77	1.98
	2	3	2.73	2.15		
	3	3	2.83	10.37		
Conventional AC	1	13	9.47	3.19	9.29	5.76
	2	3	9.71	0.70		
	3	3	8.69	10.13		
CKF Process BC	1	14	2.87	4.55	2.94	2.25
	2	3	2.96	3.93		
	3	3	3.00	2.65		
CKF Process AC	1	14	7.71	3.20	7.51	2.87
	2	3	7.55	2.65		
	3	3	7.28	4.52		
NIST Biomass A	1	14	2.64	8.01	2.50	12.64
	2	3	2.73	5.33		
	3	3	2.14	4.18		
NIST Biomass B	1	12	6.52	3.88	6.31	2.97
	2	3	6.21	1.32		
	3	3	6.18	6.67		
			Average RSD_r	**4.46**	**Average RSD_R**	**4.74**

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Next Steps

What Could It Mean For The Industry?

On a "per-facility" basis

"Typical" bioethanol production facility ALREADY operating in-situ CKF process

- ✓Assume **100 Mgal/Yr** capacity
- ✓Assume **1% cellulosic ethanol** based on Neogen methodology
- ✓Assume **$1.60** D6-D3 RIN spread

Predicts ~$1.6m annual benefit with no operational/manufacturing modification before:

- ✓YDC license fee
- ✓Analytic lab fees
- ✓Additional audit costs

What Could It Mean For The Industry?

Using LCFS to approximate the quantity of CKF processes in situ today

Phase 1 – Facilities that have already adopted cellulase-assisted fermentation technology:

- ✓Ethanol production capacity approved in the LCFS program is **~5.8Bgal**
- ✓Assume **1% cellulosic ethanol** based on Neogen methodology
- ✓Assume **$1.60** D6-D3 RIN spread

Predicts ~$93m uplift for those plants on a no-change basis

Phase 2 – Facilities that currently utilize conventional fermentation technology:

- ✓Ethanol production capacity NOT approved in the LCFS program is **~10.5Bgal**
- ✓Same assumptions as above

Predicts potential ~$168m D3 RIN “carrot”

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How Do We Get There?

2021
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Sep-Oct E48.05 Sub-, E48 Main committee ballot 2023

FELC 2023
FUEL ETHANOL LABORATORY CONFERENCE

ASTM INTERNATIONAL

2023
Nov-Dec
ICOS review and ASTM approval

ASTM ILS 2024-2025

Research article publication Jan 2024

EPA approval Mar 2024

EPA

How Will It Work?

Phase 1

Producer objective: Prepare for facility registration by EPA

E-RDYDC

Tasks:

- Purchase E-RDYDC under supply agreement and perform in-house analysis or outsource to your analytical partner
- Gather % cellulosic ethanol data from multiple fermentations
- Plant fermentation process development (if required)
- Predict % cellulosic ethanol

https://www.epa.gov/fuels-registration-reporting-and-compliance-help/how-register-new-renewable-fuel-producer-renewable

Phase 2

Producer objective: Generate D3 RINs

E-YDC

Tasks:

- Order YDC ($FOC) under supply agreement and perform in-house analysis or outsource to your analytical partner
- Generate % cellulosic ethanol data from multiple fermentations
- Complete 3rd party engineering audit and submit to EPA for facility registration
- Re-submit data to EPA every 500K gallons for re-certification
- Pay a license fee to Neogen based on D3 $ premium generated on a monthly basis

https://www.govinfo.gov/content/pkg/CFR-2022-title40-vol19/pdf/CFR-2022-title40-vol19-sec80-1451.pdf - see page 4

Summary

- A **VCSB method** has been developed for the measurement of the relevant portion of **cellulosic content** as defined by the EPA
- This method is currently under consideration for adoption as a standard within **ASTM**
- Once approval has been finalized, **EPA approval** is expected to quickly follow based on existing guidance
- For those plants already generating cellulosic ethanol, conversion of the relevant D6 to D3 RINs will be possible with no operational changes required, and will deliver significant financial benefit
- For those plants not currently generating cellulosic ethanol, could this be the additional benefit needed to justify the change?!

For more information on how to join ASTM and help support the VCSB method approval,
contact David Mangan
(dmangan@neogen.com)

For more information on the commercial aspects of accessing the analytical technology,
contact Matt Nichols
(mnichols@neogen.com)

NEOGEN

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Ruth Ivory
Anna Draga
Tadas Kargelis
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Lucie Charmier
Artur Rogowski
Andrea Mascherpa

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Justin Sluiter
Katie Michel

POET
Bart Plocher
Melissa Tille

Novozymes
David Gogerty
Geoff Moxley

TCD
John O'Brien
Manuel Ruether

CCRC
Paristoo Azadi

ASTM standard collaborators
Too many to list!

Thank you for listening

Any questions?