



Developing Enzyme Systems for Converting Pretreated DDGS to Fermentable Sugars

Mike Cotta
Bruce Dien
Xin-Liang Li
Eduardo Ximenes

National Center for Agricultural Utilization
Research
Peoria, IL



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Outline

- Introduction
- Digesting DDGS w/ commercial enzyme preparations
- Using DDGS as a feedstock for producing custom hydrolytic enzymes
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Opportunity for New Enzyme Preparations

Hot-water and AFEX pretreatment of distillers grains have numerous advantages compared to acid hydrolysis.

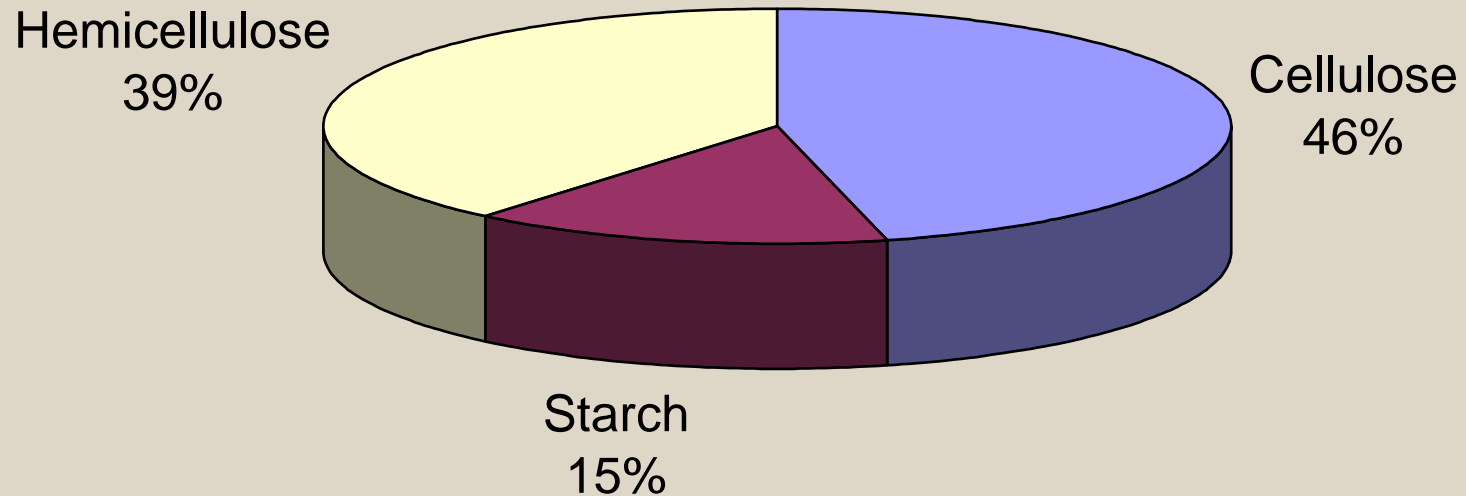
But, these treatments do not convert the xylan related carbohydrates to fermentable monosaccharide sugars



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Pentose sugars are significant



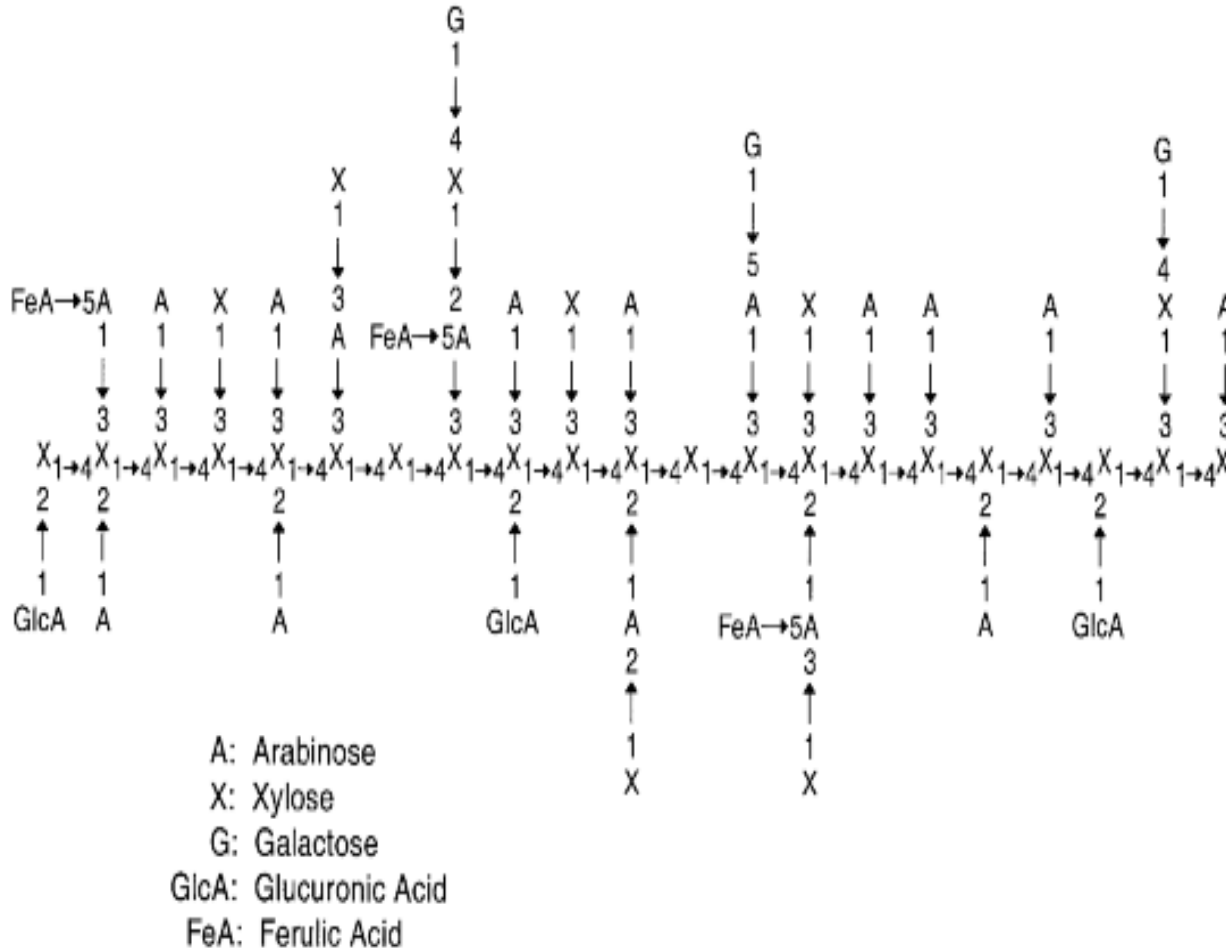
Relative Amounts of Carbohydrates



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Corn fiber heteroxylan



Saulnier



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Phenolic acids in corn fiber

	% w/w of Cell Wall
<i>p</i> -Coumaric acid	0.3
Ferulic acid	2.9
Dehydrodiferulic acids	2.5
5-5' dimer ^a	23
8-O-4' dimer ^a	35
8-8' dimer ^a	11

^a Values are expressed as % of total dimers.

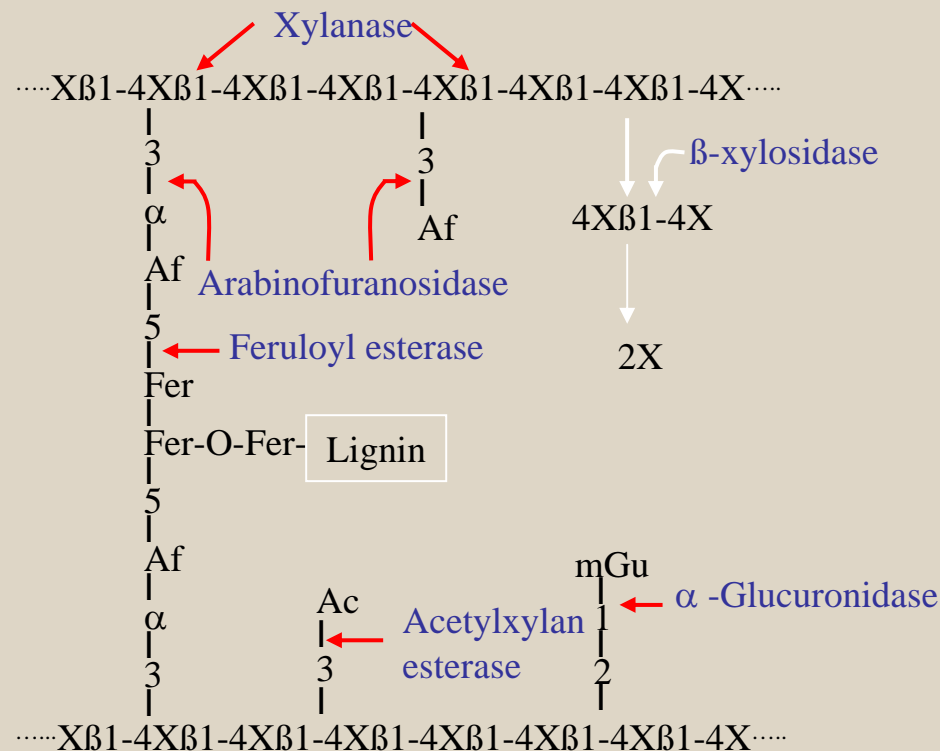
Saulnier and Thibault, 1999



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Complex Mixture of Enzymes Needed to Degrade Arabinoxylan



Selinger et al., 1996



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Research Strategy at NCAUR

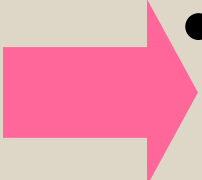
- Enzymes for digesting xylan from distillers grains' pretreated by AFEX & hot-water pretreatments
 - Commercial enzyme preparations
 - Custom enzyme supplements
 - Purified enzyme activities (when available)
- Validate ethanol fermentability of sugars using:
 - Wild-type *S. cerevisiae* for fermentation of hexoses
 - *E. coli* strain FBR5 for fermentation of pentoses



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Major Activities of Commercial Enzymes

Enzyme Prep.	Filter paper	CMC	OSX	WSX -HV	WSX-LV	WIX	CFX
	Activities Measured in IU/ml						
Novo188	8.50	481	123	118	224	48.9	55.9
Spezyme CP	39.0	4277	2622	3022	3898	3635	506
GC 220	110.0	5185	2782	3955	4480	2258	581
Multifect Xylanase	0.77	30	25,203	na	na	na	na

Abbreviations:

Xylans: OSX: oat spelt xylan; WSX-HV: wheat soluble xylan, high viscosity; WSX-LV: wheat soluble xylan, low viscosity; WIX: wheat insoluble xylan; CFX: corn fiber arabinoxylan



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Debranching activities

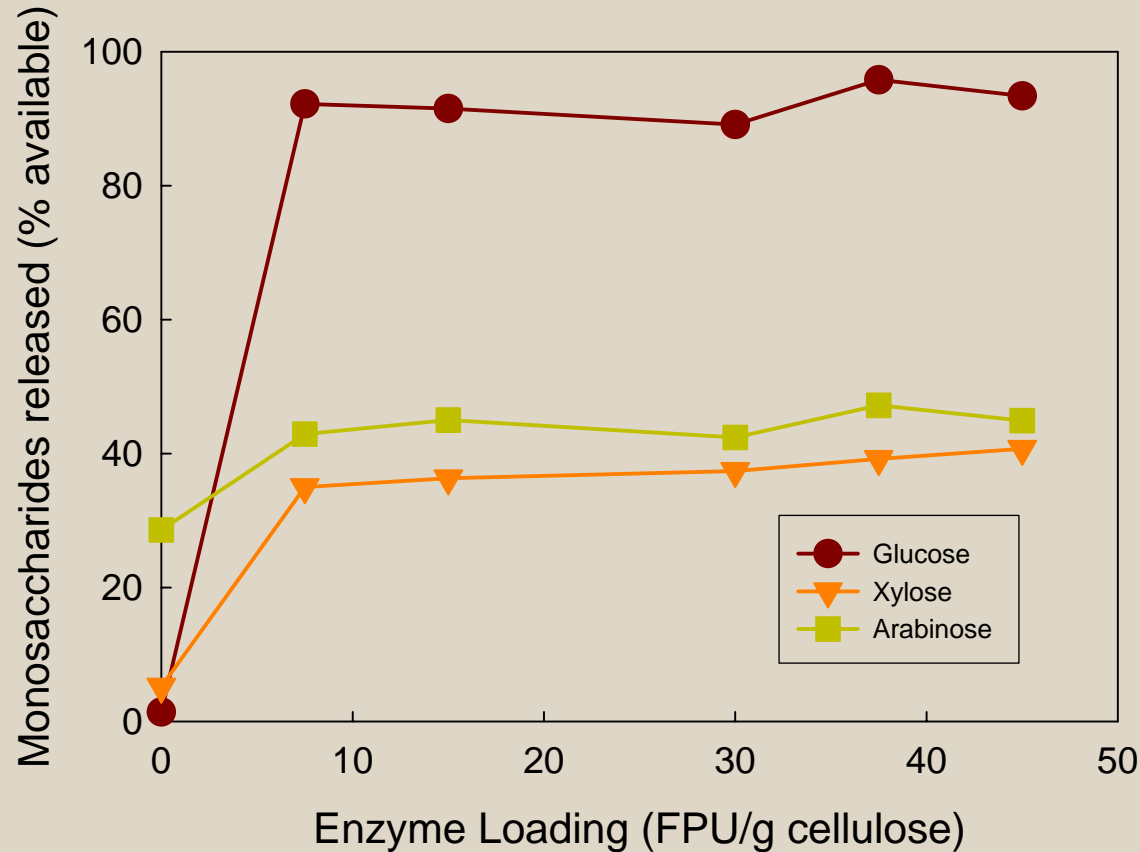
Enzyme Prep.	Arabinosidase	β -glucosidase	β -xylosidase	α -galactosidase	feruloyl esterase
	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
Novo 188	29.3	665	16.6	116	0.60
Spezyme CP	22.6	128	7.30	0.39	0
GC 220	3.06	99.7	23.3	3.90	0
Multifect Xylanase	9.44	35.9	22.6	2.39	0



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I. Effectiveness of using a cellulase preparation on hot-water treated DDGS



Just adding GC220 Cellulase & Novo188 (40 U/g cellulose)



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II. Combining cellulase prep. w/ xylanases & hemicellulases

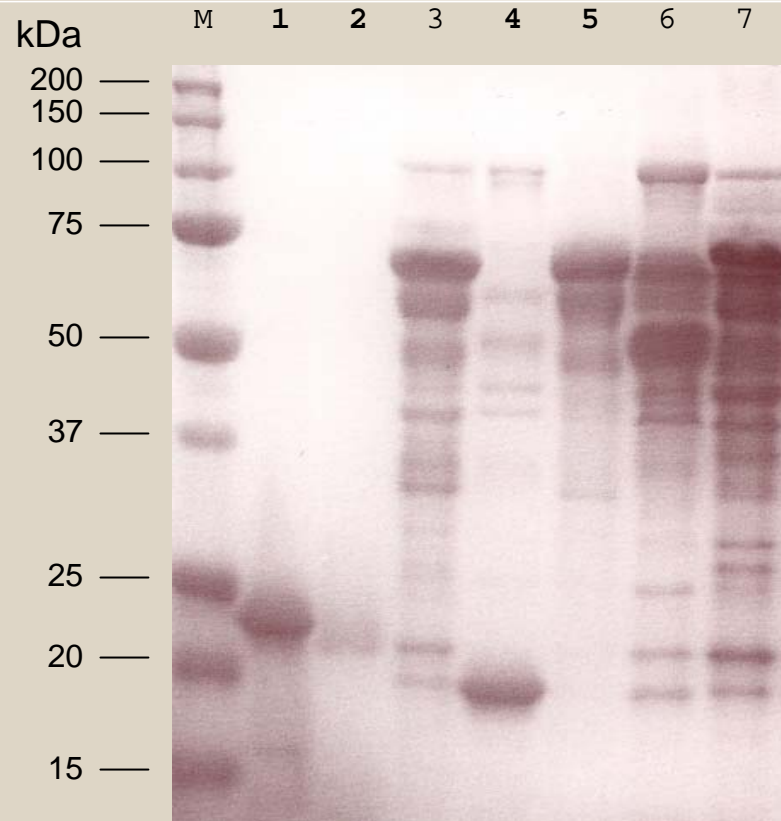
- **Endo-xylanases** (emphasizes endo-xylanase activity)
 - Multifect 720
 - GC260
 - Multifect XL
 - Multifect Xylanase
- **Hemicellulases**
 - Multifect A40
 - Optimash BG
 - Optimash XL



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Protein Gel



SDS-PAGE analysis of enzyme preps : **1, Multifect 720**; **2, GC260**; **3, Multifect A40**; **4, Multifect XL**; **5, Multifect xylanase**; **6, Optimash BG**; and **7, Optimash XL**.

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Digestion Assay

1. Treat DDGS at 15% solids at 160°C for 20 min
2. Add GC220 (15 FPU/g cellulose) + Novo188 (40 U/g cellulose) + binary combination of xylanase and hemicellulase as listed on next slide
3. React at 50°C & pH 4.8 (citric acid buffer) for 72 hr
4. Measure beginning and final monosaccharide yields



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Evaluating Genencor Enzymes

Binary Design: enzymes added in combination w/ cellulases

	<u>Hemicellulase</u>			
	<u>None</u>	<u>GHC-1</u>	<u>GHC-2</u>	<u>GHC-3</u>
<u>Xylanase</u>	reaction number			
None	0			
GX-1		1	2	3
GX-2		4	5	6
GX-3		7	8	9

Note: enzymes added at 25% loading of GC220 & Novo188 also added to each



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Results and conclusions

<u>Xylanase</u>	<u>Hemicellulase</u>	<u>Glucose</u>	<u>Xylose</u>	<u>Arabinose</u>
(Yields as % available)				
none	none	92.0%	37.9%	47.0%
GC260	Multifect A40	90.2%	34.8%	46.5%
GC260	Optimash BG	95.5%	40.1%	47.8%
GC260	Optimash XL	92.4%	38.0%	47.7%
Multifect XL	Multifect A40	92.3%	35.9%	47.0%
Multifect XL	Optimash BG	95.8%	40.4%	45.8%
Multifect XL	Optimash XL	95.1%	35.7%	43.8%
Multifect Xylanase	Multifect A40	99.0%	43.9%	48.3%
Multifect Xylanase	Optimash BG	96.8%	42.4%	48.8%
Multifect Xylanase	Optimash XL	97.5%	43.0%	47.3%

Conclusion: Multifect Xylanase combinations were most effective.



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III. Separating effect of Multifect Xylanase from Hemicellulases

Factorial Design

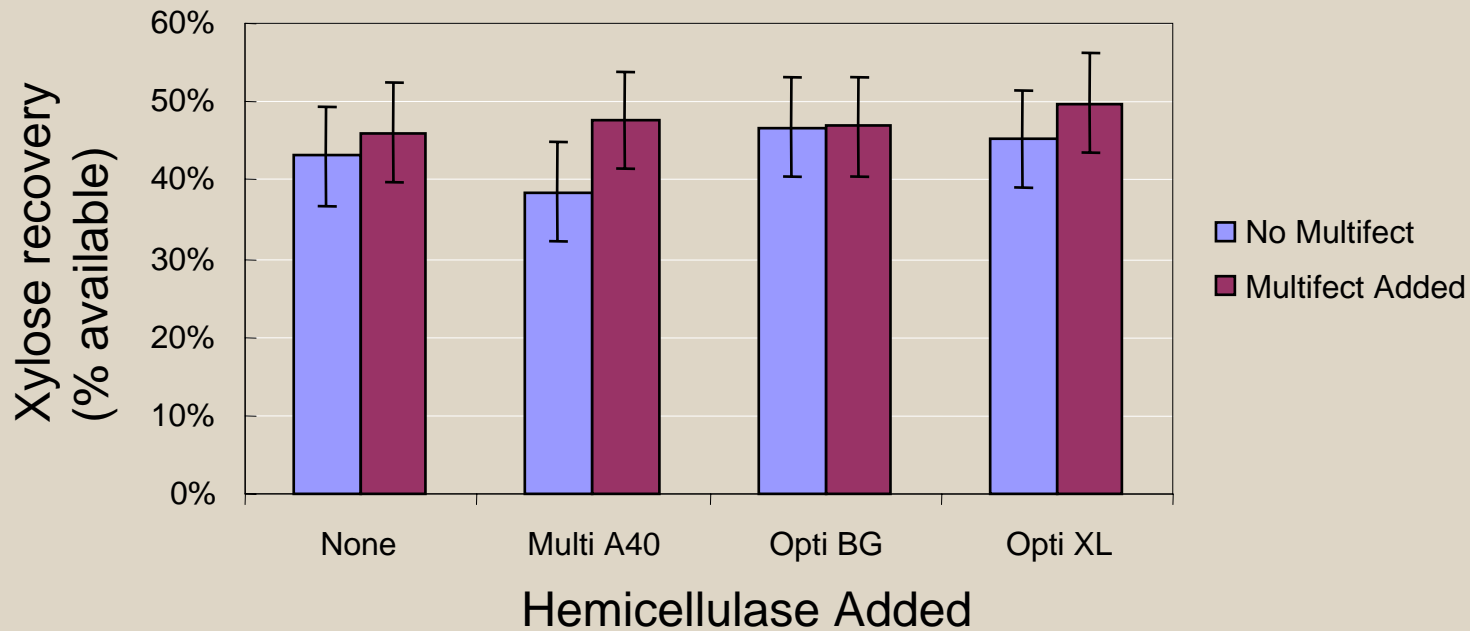
Levels/Factors:	Xylanase	Hemicellulase
1	None	None
2	Multifect Xylanase	Multifect A40
3	NA	Optimash BG
4	NA	Optimash XL



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Resolving effects of HC's and Multifect



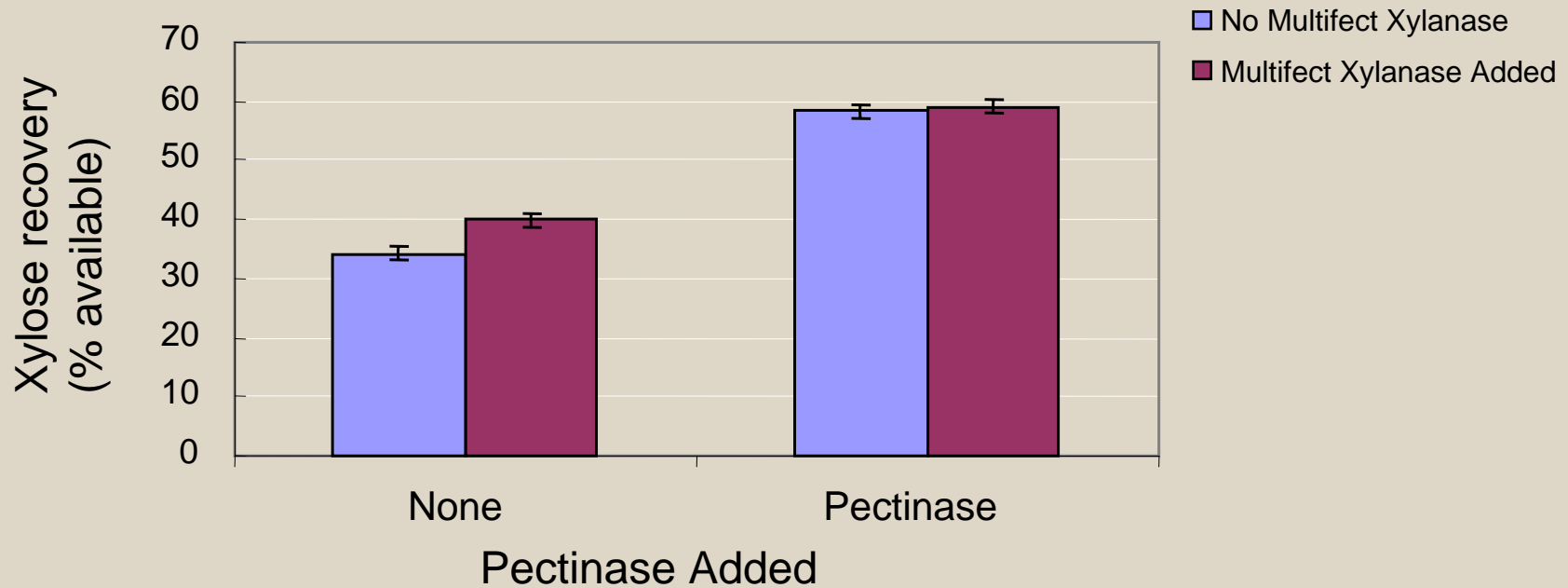
Conclusion: multifect xylanase increases xylose and glucose yields independent of hemicellulase. (glucose data not shown)



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Effect of adding pectinase



Conclusion: adding pectinase increased xylose and arabinose (data not shown) yields.



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Conclusions for digestion work

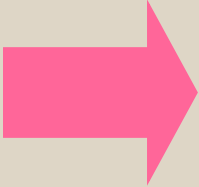
- ❖ Multifect Xylanase and Optimash BG were most effective against HW-DDGS
- ❖ While monosaccharide yields of arabinose and xylose were approx. 50%, almost all of the xylan was dissolved w/ Multifect Xylanase (>90%)
- ❖ Further experiments will focus on varying enzyme loadings, pretreatment time, and applying custom enzyme preparations



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Flask Fermentation Protocol

Microorganisms: *T. reesei*, *A. niger*,
and *Aureobasidium pullulans*

- Flasks: 250 ml baffled Erlenmeyer Flasks
- Inoculum: 5%v/v of a culture pre-grown on PD or YM
- Media: 50 mL of 40 g/l DDGS w/ either CSL+ minerals or YM
- Culture Conditions: 30°C & 250 rpm for 8 days
- Sampled for: xylanase activity daily and for other activities at days 4 and 8



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A. DDGS vs. Beet pulp by *A. niger*

<u>Carbon Source</u>	<u>Corn Steep Liquor</u>	<u>Xylanase</u>	<u>Cellulase</u>	<u>Arabino-furano side</u>	<u>Xylosidase</u>	<u>Glucosidase</u>	<u>FAE</u>
		IU/ml	IU/ml	IU/ml	IU/ml	IU/ml	IU /ml
Beet pulp	yes	26.0	2.74	5.70	0.49	4.3	0.27
DDGS	no	35.3	2.50	1.79	0.82	3.6	0.32
DDGS	yes	40.2	2.69	3.01	0.90	3.2	0.33

Conclusion: enzyme profile for DDGS is comparable with Beet Pulp



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B. Which is better DDGS or DDG?

Trichoderma reesei and *Aspergillus niger* enzyme activities after 8 days growth in DDG and DDGS corn fractions

	Filter paper (50 mg) (IU/ml)	CMC (1%) (CMCase IU/ml)	Oat spelt xylan (1%) (xylanase IU/ml)	Corn Fiber Arabinoxyl an (1%) (xylanase IU/ml)	ρ -NPG (2.5 mM) (β - glucosida se IU/mL)	ρ -NPA (2.5 mM) (α - arabinofuranosi dase IU/mL)	ρ -NPX (2.5 mM) (β -xylosidase IU/mL)
<i>A.niger</i> (DDGS)	0.270	5.39	53.8	4.65	2.24	0	1.14
<i>A.niger</i> (DDG)	0.216	4.85	68.2	5.06	2.04	0	1.44
<i>T.reesei</i> (DDGS)	0.407	4.20	73.5	3.00	0.135	0.309	1.16
<i>T.reesei</i> (DDG)	0.355	4.70	33.74	2.30	0.325	0.340	0.825

Conclusion: DDGS gave either similar or superior results to DDG for both strains tested.

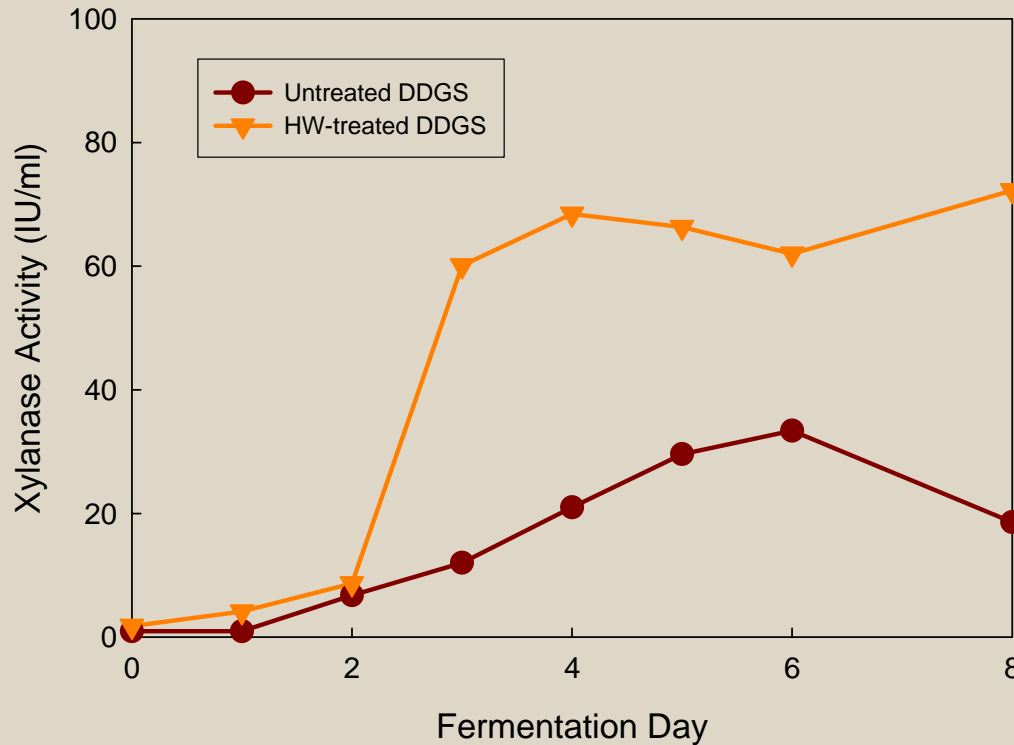


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C. Hot-water vs. Untreated DDGS for *A. niger*

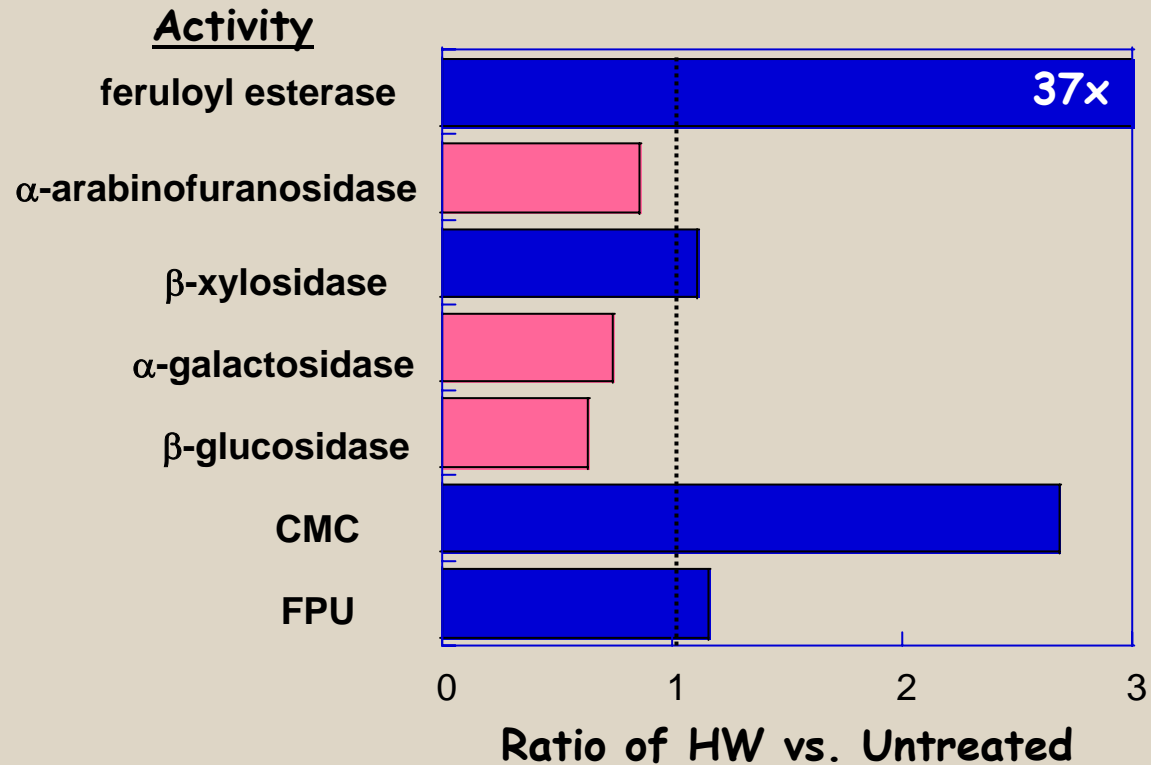
A. niger Fermentation Xylanase Production



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C. Hot-water vs. Untreated DDGS for *A. niger*



Conclusion: enzyme titers from HW-DDGS cultures were superior or comparable to untreated DDGS



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D. Available Purified Hydrolytic Enzymes

Enzyme ^a	Size (AA)	Binding ^b Sequence	pH Range	Optimum Range (°C)	Best Temp.
CelA	459	NCDD		4.3-6.8	30-50
CelB	471	NCDD		4.8-7.6	30-50
CelC	449	NCDD		4.6-7.0	30-45
CelD	455	NCDD		4.5-7.0	30-50
CelE	477	NCDD		4.5-7.5	30-50
CelF	432	CBD		4.5-6.5	30-50
CelG	Partial	None		4.5-7.0	30-50
CelH	491	NCDD		ND	ND
CelI	495	NCDD		ND	ND
CelJ	Partial	NCDD		ND	ND
BglA	663	None		5.5-8.0	45-55
XynA	362	NCDD		5.5-7.5	50-60
LicA	245	None		4.5-8.0	35-55
ManA	574	CBD/NCDD		ND	ND
AxeA	313	None		7.0-8.5	30-45
FaeA	330	None		5.5-8.0	30-50

^aCel, cellulase; Bgl, beta-glucosidase; Xyn, xylanase; Lic, beta-glucanase (lichenase); Man, mannanase; Axe, acetylxyylan esterase; and Fae, feruloyl esterase. ^bNCDD, non-catalytic docking domain.

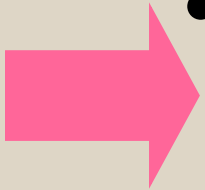


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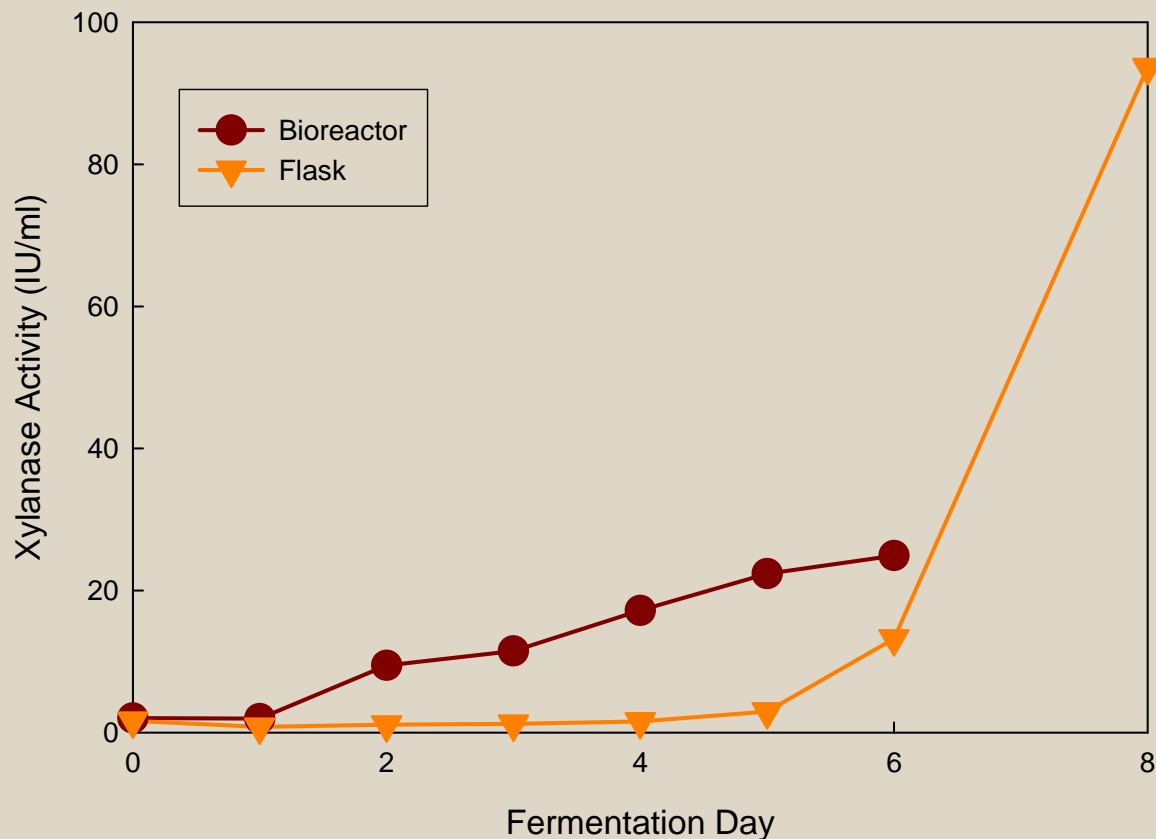


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I. Culturing *T. reesei* in bioreactor

Comparison of *T. reesei* growing in Flask vs. Bioreactor

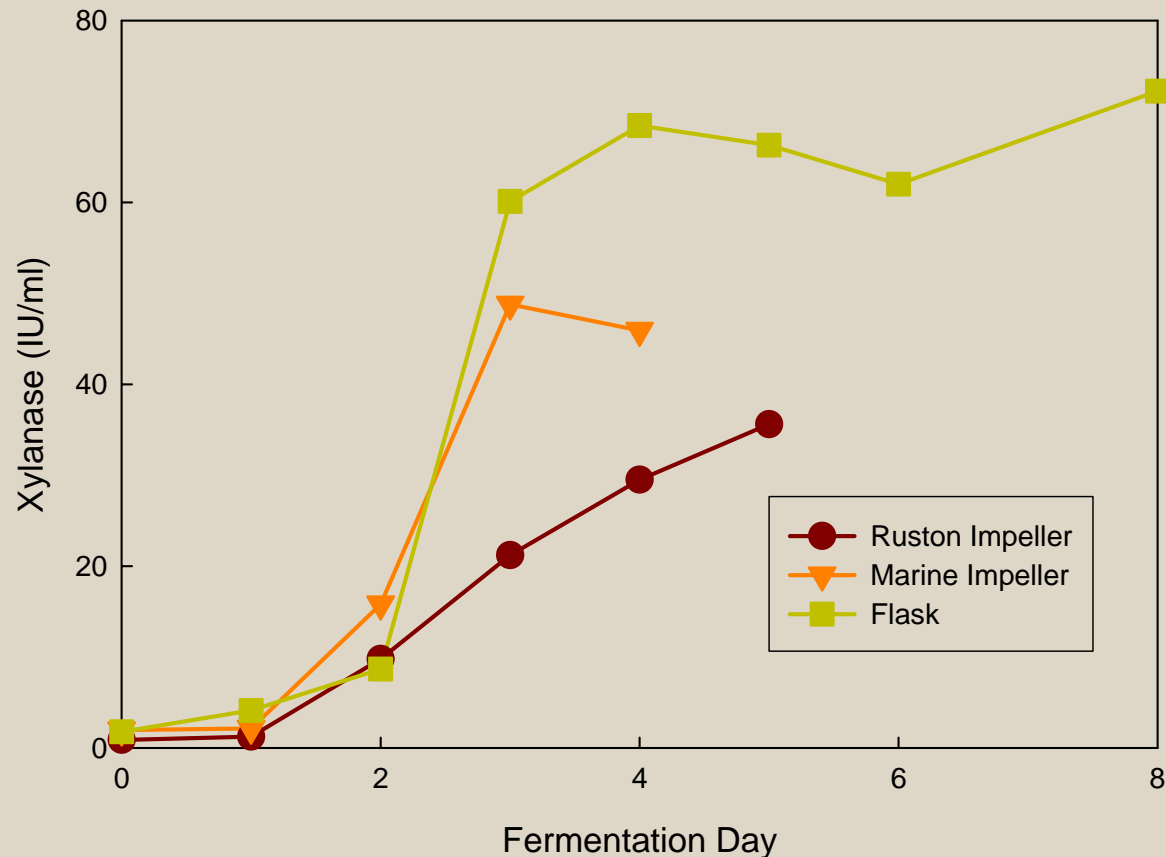


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II. Culturing *A. niger* in bioreactor

Effect of Impeller Design on *A. niger* Fermentation & Comparison to Flask Culture



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Summary

- A combination of cellulase and xylanase preparations releases 96% of the glucose, 50% of the xylose, and 54% of the arabinose
- A wide range of enzyme activities can be produced by growing various fungi on HW-DDGS
- Production of enzymes on DDGS is amenable for culturing in laboratory sized bioreactors



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