Sugar-based Ethanol Biorefinery: Ethanol, Succinic Acid and By-Product Production

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DELIVERABLE REPORT (FINAL)
To
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LSU AGRICULTURAL CENTER
AUDUBON SUGAR INSTITUTE
FINAL REPORT FOR GRANT # DE-FG36-05GO8007
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Project Objectives and Strategy

This project focused on increasing the number of marketable product options for raw sugar mill based industries through a biorefinery alternative that converts biomass (i.e. bagasse, CLM[cane leaf matter] and molasses) to ethanol and biochemicals. This project addresses some of the major technical problems required to bring to commercialization a sugar based biorefinery. These are:

1) Develop an appropriate and scaleable pretreatment, hydrolysis and fermentation process for production of ethanol from bagasse and CLM using available supplements.
2) Identification of potential by-products that can be produced from biorefinery process
3) Investigation into the effects of a biorefinery model on the economics of a raw sugar mill by creating an economic model for a Louisiana sugar mill that can be modified with "add-on" technologies to produce a biorefinery that produces both sugar and ethanol from sugar cane.

Strategy

The major objective is to assess the respective merits of several pretreatments on bagasse and CLM with the goal of converting these feedstocks to ethanol using a fermentation platform. The strategy is as follows:

- To develop appropriate and scaleable fermentation processes for production of ethanol from bagasse, CLM, and molasses,
- To investigate other products that can be produced to supplement income from ethanol,
- To investigate potential for strain improvement to allow co-fermentation of glucose and xylose,
- To investigate the various "waste streams" for saleable products,
- To investigate the potential for anaerobic treatment of alcohol stillage from biomass fermentations.

Table of Contents

EXECUTIVE SUMMARY

The work conducted in this project is an extension of the developments itemized in DE-FG-36-04GO14236. This program is designed to help the development of a biorefinery based around a Raw Sugar Mill, which in Louisiana is an underutilized asset. Some technical questions were answered regarding the addition of a biomass to ethanol facility to existing sugar mills. The focus of this work is on developing technology to produce ethanol and valuable by-products from bagasse. Three major areas are addressed, feedstock storage, potential by-products and the technology for producing ethanol from dilute ammonia pre-treated bagasse.

Sugar mills normally store bagasse in a simple pile. During the off season there is a natural degradation of the bagasse, due to the composting action of microorganisms in the pile. This has serious implications if bagasse must be stored to operate a bagasse/biorefinery for a 300+ day operating cycle. Deterioration of the fermentables in bagasse was found to be 6.5% per month, on pile storage. This indicates that long term storage of adequate amounts of bagasse for year-round operation is probably not feasible.

Lignin from pretreatment seemed to offer a potential source of valuable by-products. Although a wide range of phenolic compounds were present in the effluent from dilute ammonia pretreatment, the concentrations of each (except for benzoic acid) were too low to consider for extraction. The cellulosic hydrolysis system was modified to produce commercially recoverable quantities of cellobiose, which has a small but growing market in the food process industries. A spin-off of this led to the production of a specific oligosaccharide which appears to have both medical and commercial implications as a fungal growth inhibitor. An alternate use of sugars produced from biomass hydrolysis would be to produce succinic acid as a chemical feedstock for other conversions. An organism was developed which can do this bioconversion, but the economics of succinic acid production were such that it could not compete with current commercial practice.

To allow recovery of commercial amounts of ethanol from bagasse fermentation, research was conducted on high solids loading fermentations (using *S. cerevisiae*) with commercial cellulase on pretreated material. A combination of SHF/SSF treatment with fed-batch operation allowed fermentation at 30% solids loading. Supplementation of the fermentation with a small amount of black-strap molasses had results beyond expectation. There was an enhancement of conversion as well as production of ethanol levels above 6.0% w/w, which is required both for efficient distillation as well as contaminant repression. The focus of fermentation development was only on converting the cellulose to ethanol, as this yeast is not capable of fermenting both glucose and xylose (from hemicellulose). In anticipation of the future development of such an organism, we screened the commercially available xylanases to find the optimum mix for conversion of both cellulose and hemicellulose. A different mixture than the spezyme/novozyme mix used in our fermentation research was found to be more efficient at converting both cellulose and hemicellulose. Efforts were made to select a mutant of *Pichia stipitis* for ability to co-ferment glucose and xylose to ethanol. New mutation technology was developed, but an appropriate mutant has not yet been isolated. The ability to convert to stillage from biomass fermentations were determined to be suitable for anaerobic degradation and methane production. An economic model of a current sugar factory was developed in order to provide a baseline for the cost/benefit analysis of adding cellulosic ethanol production.

TASKS

Feedstock Characterization

Bagasse is the residual plant material left after milling sugarcane and extracting the juice. As it leaves the mill it contains about 50% moisture. Approximately 200lbs is produced per ton of sugarcane processed. Of this material between 75 and 85% is burned directly to produce steam to power the sugar mills. The residual is normally pile stored near the mills until it can be removed. CLM is the leaf matter that is removed from the cane during harvesting. By weight it about the same as the bagasse (200lbs/ton on cane). Some of this must stay on the fields to improve tilth, but approximately 50% can be removed without harm. Both bagasse and CLM have been identified as potential feedstocks for ethanol production in a biorefinery.

The composition of sugarcane bagasse from four Louisiana mills (Alma, Cora Texas, Raceland and Enterprise) is summarized in Table 1. Bagasse constituents ranged from 3.71% - 6.22% (ash); 19.07% - 21.98% (total lignin); 40.38% - 43.19% (glucan); and 22.60% - 25.06% (xylan).

Table 1. Composition of Bagasse from four Louisiana sugarcane mills.

	Percent (Total Dry Mass) *					
Biomass constituent	**NIST Values**	**NIST Control**	**Alma**	**Cora Texas**	**Raceland**	**Enterprise**
Ash	4.0 ± 0.5	3.55	3.71	3.85	6.22	4.32
Extractives	4.4 ± 5.3	2.09	2.2	2.09	1.90	2.18
Acid Soluble Lignin (ASL)	2.0 ± 2.0	4.19	3.74	4.43	4.01	4.31
Acid Insoluble Lignin (AIL)	22.3 ± 2.5	19.54	15.99	17.55	15.06	17.43
Glucan	40.2 ± 3.2	40.08	41.39	43.19	40.38	41.36
Xylan	21.5 ± 3.1	23.28	25.06	23.41	22.60	23.00
Arabinan	1.8 ± 0.5	1.89	2.53	1.49	2.46	2.50
Mannan	0.4 ± 0.4	ND	0.39	ND	ND	ND

*= NIST, Reference values provided by the National Institute of Standards & Technology; NIST Control, was run as an internal standard.

Table 2. Composition of Bagasse, Bagasse Pith, and CLM (based on dry weight)*

	Glucan %	**Xylan %**	**Galactan %**	**Arabinan %**	**Ash %**	**Extractive %**
CLM	27.09	20.01	1.44	2.34	5.7	3.2
Bagasse	34.6	18.49	00.45	0.95	6.5	2.4
Bagasse pith(first batch)	19.7	12.32	0.57	0.95	25	1.9
Bagasse pith (second batch)	26.28	14.37	1.13	1.3	16	2.36

*Obtained during DOE Grant DE-FG-36-04GO14236

Feedstock- Changes on Storage- Sugar mills normally store bagasse in a simple pile. During the off season there is a natural degradation of the bagasse, due to the composting action of microorganisms in the pile. This has serious implications if bagasse must be stored to operate a bagasse/biorefinery for a 300+ day operating cycle. Fresh bagasse would be available for significantly less operational time. There was little or no data available on bagasse deterioration in terms of loss of cellulosic ethanol potential.

A box was constructed to store bagasse for a year (December 2006 –December 2007) (Figure 1). Moisture and temperature were monitored using automatic data loggers. Composition analysis was conducted using the wet chemistry methods recommended by the National Renewable Energy Laboratory (NREL). The bagasse was monitored remotely, initially on an hourly basis, for relative humidity and temperature. In the first month the bagasse pile saturated from the winter rains in Louisiana, and did not dry out for the 12 month period of the test. The internal temperatures of the pile stayed within the 35-45^{o}C range (ideal for microbial action). A portion of the monitored profile is given in Figure 2. The moisture content of the bagasse was determined for the last four months of the test. It maintained a fairly constant level of about 83.3% on weight (Table 3).

Figure 1. Bagasse Storage Box at the Beginning of the Experiment.

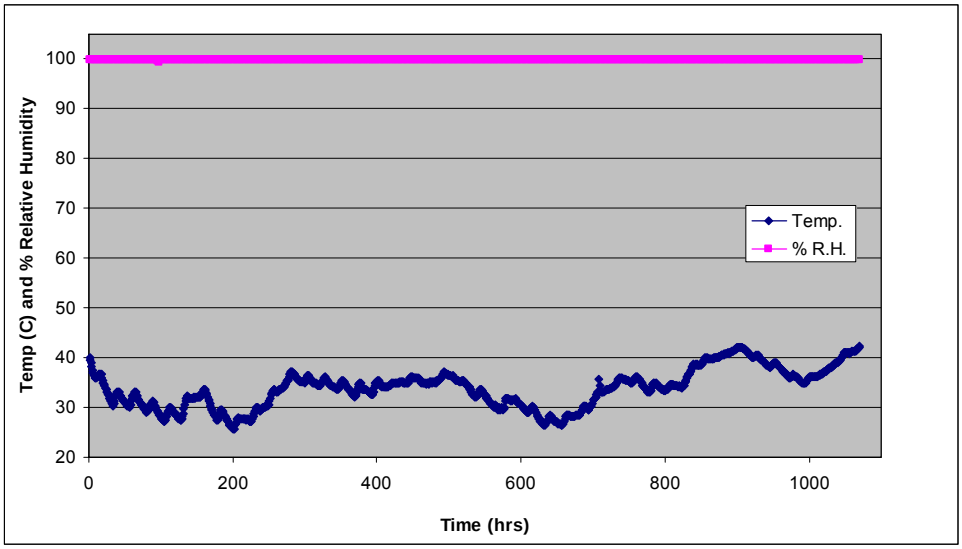


Figure 2. Humidity (%) and temperature (^{o}C) as a function of time (hrs).

Table 3. Moisture in the non-aerated bagasse pile.

Month	% Moisture
9	83.84
10	83.97
11	84.13
12	81.33

The results of changes in a non-aerated pile are presented in Table 4 as they are the most relevant to biorefinery operations. Ash levels (washed samples) were used to monitor weight change in the bagasse as there was no provision for direct weighing of the pile. Although not perfect, it is an adequate estimate as the ash (inorganic) is the one component which will not to change due to biological deterioration. All composition analyses were conducted using NREL standard methodology.

Table 4. Composition changes in bagasse with storage.

Data (Time (months)	(% dry weight)			
	Glucan %	Xylan %	Lignin %	Ash %
start	40.27	21.33	29.64	7.02
1	40.88	19.82	31.25	5.67
7	35.72	18.94	29.44	8.75
8	34.25	18.71	28.60	9.05
9	32.93	12.70	27.77	15.38
10	33.70	12.70	26.26	14.32
11	25.00	12.66	27.30	19.73
12	24.79	15.67	29.67	26.68

The estimated rate of loss of feedstock material(glucan, xylan) is about 6.6% per month (Table 5).

Table 5. Rate loss of feedstock material under non-aerated storing conditions.

Component	Loss rate percent/month
Glucan	6.90
Xylan	6.30
Lignin	6.25

Theoretical weight loss estimate, determined from changes in ash content was 5.8% per month of storage (Figure 3). Figure 4 shows the bagasse pile at the end of the study.

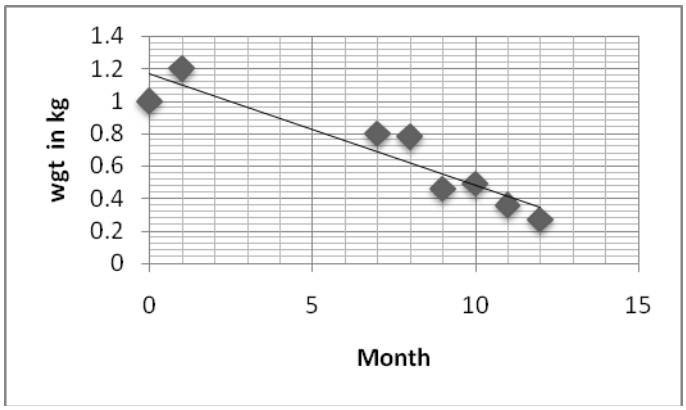


Figure 3. Estimated Dry Weight Loss Based on Increased in Ash Content in Samples. The rate of loss from one kg dry bagasse is about 60 g/month.

Figure 4. Bagasse Pile at the End of One Year.

It is obvious that pile storage is not optimum for a bagasse processing biorefinery. Practically bagasse should probably be stored for no more than 2 months. Under roof storage may slow the rate of deterioration (unproven), preventing the water saturation of the biomass, which produced ideal conditions for biodegradation by microorganisms, but the volume of bagasse required for yearly operation of a biorefinery would probably make this cost solution prohibitive.

Pretreatment

Under the auspices of a previous grant (DE-FG-36-04GO14236) AFEX pretreatment of bagasse and CLM was tested and optimum conditions determined. Because further research demanded volumes of pre-treated bagasse that could not be met with the available size AFEX reactors, a dilute ammonia

process was developed as an AFEX stimulant. Other methods for pretreatment were also tested, but from the point of view of extractable by-products from the lignin components. These results will be reported under by-products.

Dilute Ammonia Treatment

Ammonium hydroxide, steam and water treated biomass contained 56.6% glucan, 63.6% glucan, and 60.5% glucan, respectively (Table 6). Less than 1% sugars (i.e. glucose, xylose, mannose, arabinose, and cellobiose) were lost in solution post treatment.

Table 6: Composition Analysis of water, steam and ammonium hydroxide treated biomass processed at 160º C for 1 h.

Biomass constituent (%)	NIST Values	NIST* QC	Raw Bagasse	Treatment		
				Water 160 ºC	Steam 160 ºC	0.02 g NH_3^+/ 100 g water 160 ºC
Ash	4.0 ± 0.5	3.8	4.2	2.5	3.7	2.8
Ethanol extractives	4.4 ± 5.3	2.5	1.1	1.7	4.0	3.9
Acid soluble Lignin	2 ± 2	5.7	5.3	3.9	4.5	5.0
Acid insoluble Lignin	22.3 ± 2.5	23.6	19.7	20.4	18.1	16.1
Glucan	40.2 ± 3.2	39.1	38.4	60.5	63.6	56.6
Xylan	21.5 ± 3.1	22.4	24.1	13.3	8.4	24.0
Arabinan	1.8 ± 0.5	1.5	1.9	0.3	0.0	1.2
Mannan	0.4 ± 0.4	1.0	0.0	1.1	1.9	1.7

*= NIST quality control bagasse.

Treatment of sugarcane bagasse with dilute ammonium hydroxide (0.5g ammonium hydroxide (28% as NH3)/g dry biomass) at 160 ºC for 60 min was selected as the process to be used in the production of ethanol and stillage. Approximately, 16 kg of alcohol stillage were produced per pilot run in a 20 L reactor.

Simultaneous Saccharification and Fermentation

SSF were conducted at 10% and 30% loading. Table 7 shows the ethanol yields and % conversion (based on glucan composition) achieved in a 72 hr fermentation.

Table 7: High solids fermentations, treated bagasse. Ethanol produced and % glucan conversion shown.

	10% Biomass Loading		30% Biomass Loading	
Treatment	**Ethanol %**	**Conversion %**	**Ethanol %**	**Conversion %**
dilute ammonia	2.4	84	3.6	42.4
water	1.2	39	2.8	30.8

This data illustrates two points. First, temperature alone does not deconvolute biomass (as illustrated by the low conversion with water treatment). Second, the drop in conversion at high solids loading points to end product inhibition of the cellulases. This leads to the concept that a fed-batch fermentation may be more effective than a straight 30% solids loading for bioconversion of bagasse.

By-Products

Lignin Extractibles

A number of different pretreatments of bagasse were tested for their ability to produce phenolic compounds from lignin (Table 8). The total extracted phenolic concentrations were determined as a function of pretreatment.

Table 8. Phenols extracted from bagasse, CLM, and pith with post caustic, organosolv and oxidant treatments.

Biomass	Treatment (% chemical wt/wt dry biomass)	Total Phenols (mg vanillin g^{-1} dry biomass)	Phenols Extracted* (mg.g^{-1} dry biomass)
Bagasse	5% NaOH	30.2	1.3
	5% NaOH=>5% NaOH	48.9	1.0
	50% ethanol	6.7	1.0
	50% ethanol =>5% NaOH	28.8	0.8
	50% ethanol & 5% NaOH	27.9	2.7
	50% ethanol & NaOH => 5% NaOH	30.7	0.7
	10% Ox-B	5.7	0.1
	10% Ox-B =>5% NaOH	46.7	0.7
	10% Ox-B & 50% ethanol	8.0	0.7
	10% Ox-B & ethanol => 5% NaOH	44.7	0.9
CLM	5% NaOH	47.0	0.1
	5% NaOH => 5% NaOH	40.0	0.1
	50% ethanol & 5% NaOH	42.8	1.1
	50% ethanol & 5% NaOH => 5% NaOH	30.5	0.1
Pith	5% NaOH	31.4	2.0
	5% NaOH => 5% NaOH	30.8	1.2
	50% ethanol & 5% NaOH	27.6	4.2
	50% ethanol & 5% NaOH => 5% NaOH	44.0	0.6

* Phenols were extracted with dichloromethane and 3-phenylphenol was used as an internal standard for the quantification of extracted phenols.

The various extracts were analyzed using mass spectroscopy, the major products identified and concentrations determined. Table 9 shows the identity and concentrations of the most prevalent phenols by pretreatment method, found in the washed material extracted from the biomass.

Chromatographic confirmation against standards for some of the more economically significant compounds detected are being conducted to validate their identifications. Vanillin, a phenol with significant commercial value, was found in the liquid stream from every pre-treatment. Phenols, including 2-methoxy-4 vinyl phenol, 1,1 biphenyl 2 chloro mequinols and ethanones appeared repeatedly from each treatment. They also have commercial value. Several chlorinated compounds were also tentatively identified; 2-methoxy-3,6 dichlorophenol , 4,5 dichloro 2 methoxy, phenol, and 2,4,6 trichloro, 3,5 dichloro 2 hydroxy acetophenone.

Table 9. Phenolic identification and concentration in pretreatment effluent by extraction process

Biomass	Treatment (% chemical wt/wt of dry biomass)	GC-MS Retention time (min)	Compound	Total Phenols (µg vanillin g^{-1} dry bagasse)
Bagasse	5% NaOH	6.79	benzofuran 2,3 dehydro	255.3
		8.73	2 methoxy-4-vinylphenol	616.9
		9.71	benzaldehyde, 4-hydroxy	245.1
		10.51	vanillin	87.9
	5% NaOH=> 5% NaOH	6.79	benzofuran 2,3 dihydro	297.0
		8.73	2 methoxy-4-vinylphenol	388.6
		9.79	benzaldehyde, 4-hydroxy	104.8
		10.44	vanillin	92.0
		15.50	benzaldehyde, 4-hydroxy-3,5-dimethoxy	30.3
	50% Ethanol & 5% NaOH	6.79	benzofuran, 2,3 dihydro	1385.4
		8.72	2 methoxy-4-vinylphenol	348.0
		9.79	benzaldehyde, 4-hydroxy	171.0
		10.46	vanillin	52.4
		16.78	4 hydroxy-2-methoxycinnamaldehyde	68.0
		19.99	ethyl ((2E)3-(4hydroxy-3-methoxyphenyl)-2-propenoate	160.2
	50% Ethanol & 5% NaOH => 5% NaOH	6.78	benzofuran, 2,3 dihydro	312.3
		8.71	2 methoxy-4-vinylphenol	275.6
		9.71	benzaldehyde, 4-hydroxy	38.7
		10.45	vanillin	38.1
	50% Ethanol	6.83	benzofuran, 2,3-dihydro	675.3
		8.71	2 methoxy-4-vinylphenol	42.2
		9.70	benzaldehyde, 4-hydroxy	51.3
		10.44	vanillin	19.8
		16.79	4-hydroxy-2-methoxycinnamaldehyde	27.5
	50% Ethanol => 5% NaOH	6.78	benzofuran, 2, 3 dehydro	290.3
		8.71	2 methoxy-4-vinylphenol	325.2
		9.70	benzaldehyde, 4-hydroxy	82.9
		10.44	vanillin	31.7
		17.95	p-hydroxycinnamic acid, ethyl ester	44.9

Table 9. continued

Biomass	Treatment (% chemical wt/wt of dry biomass)	GC-MS Retention time (min)	Compound	Total Phenols (µg vanillin g^{-1} dry bagasse)
Bagasse – cont'd.	10% Ox-B	7.38	phenyl acetic acid	20.6
		8.49	benzaldehyde, 2 chloro-4-hydroxy	14.6
		9.66	benzaldehyde, 4-hydroxy	39.1
		10.41	vanillin	5.6
	10% Ox-B => 5% NaOH	6.83	5 chloro, 2, 3 dehydro-1-benzofuran	233.1
		8.70	2-methoxy-4-vinylphenol	131.8
		9.67	benzaldehyde, 4-hydroxy	48.6
		10.44	vanillin	20.6
		10.86	1,1 biphenyl 4 chloro	49.1
		12.05	phenol, 4,5 dichloro 2 methoxy	1.7
		12.18	benzaldehyde, 3, 5-dichloro-2-hydroxy	5.0
		13.02	ethanone 1-(5 chloro-2-hydroxy-4 methyl phenyl)	14.5
		13.51	phenol 2 chloro 6 (1,1 dimethyl phenyl)	12.6
		14.06	3 chloro-4-hydroxy phenyl acetic acid	4.3
		14.37	5-chlorovanillin	26.1
		16.94	1,1 biphenyl 4-chloro 4-methoxy	19.7
		17.51	2-biphenyl carboxylic acid	24.0
		20.93	3,5 dichloro 4 hydroxycinnamic acid	1.0
	10% Ox-B & 50% Ethanol	6.78	5 chloro, 2, 3 dehydro-1-benzofuran	313.3
		8.72	2-methoxy-4-vinylphenol	35.1
		9.44	phenol, 2,4,6-trichloro	36.2
		9.79	benzaldehyde, 4-hydroxy	119.5
		10.45	vanillin	23.4
		10.87	1,1 biphenyl 4 chloro	14.7
		12.06	phenol, 4, 5 dichloro-2-methoxy	3.8
		12.22	benzaldehyde, 3, 5-dichloro-2-hydroxy	2.5
		14.41	5-chlorovanillin	1.0
		15.49	benzaldehyde, 4-hydroxy-3,5-dimethoxy	55.7
		15.84	ethyl 3,5 dichloro 4 hydroxybenzoate	4.0
Bagasse	10% Chem Ox-B & 50% Ethanol	16.11	3,5 dichloro-4-hydroxybenzoic acid	2.1
		16.78	4-hydroxy-2-methoxycinnamaldehyde	62.4
		16.90	4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol	31.1
		17.61	1,1 biphenyl 2,3,6 trichloro	9.8

Table 9. continued

Biomass	Treatment (% chemical wt/wt of dry biomass)	GC-MS Retention time (min)	Compound	Total Phenols (µg vanillin g^{-1} dry bagasse)
Bagasse – cont'd.	10% Ox-B & 50% Ethanol => 5% NaOH	8.71	2 methoxy-4-vinylphenol	375.6
		9.44	phenol, 2,4,6-trichloro	6.9
		9.71	benzaldehyde, 4-hydroxy	58.3
		10.44	vanillin	37.9
		10.86	1,1 biphenyl 4 chloro	46.1
		11.25	ethanone 1-(2-hydroxyphenyl)	59.8
		11.47	phenol 2-methoxy, 5,1-propenyl-E	36.3
		11.81	2,3-dimethoxybenzyl alcohol	97.5
		12.05	phenol, 4,5 dichloro, 2 methoxy	3.1
		12.18	benzaldehyde, 3, 5-dichloro-2-hydroxy	11.2
		13.02	ethanone 1-(5 chloro-2-hydroxy-4 methyl phenyl)	19.9
		13.52	phenol 2 chloro 6 (1,1 dimethyl phenyl)	5.0
		14.38	5-chlorovanillin	2.7
		15.47	benzaldehyde, 4-hydroxy-3,5-dimethoxy	73.8
		16.76	4 hydroxy-2 methoxy cinnamaldehyde	11.8
		16.94	1,1 biphenyl 4-chloro 4-methoxy	24.6
		17.71	1,1 biphenyl 2,3,4 trichloro	1.4
CLM	5% NaOH	8.72	2 methoxy-4-vinylphenol	13.5
		9.66	benzaldehyde, 4-hydroxy	30.4
		10.44	vanillin	35.3
	5% NaOH => 5% NaOH	4.54	phenol, 2-methoxy	3.3
		9.66	benzaldehyde, 4-hydroxy	6.7
		10.44	vanillin	27.3
		15.49	benzaldehyde, 4 hydroxy-3,5-dimethoxy	6.9
		16.82	ethanone, 1-(4 hydroxy-3,5-dimethoxyphenyl)	7.0
		19.93	ethyl ((2E)3-(4hydroxy-3-methoxyphenyl)-2-propenoate	10.2
CLM	50% Ethanol & 5% Chem NaOH	6.78	benzofuran, 2,3 dihydro	374.9
		8.73	2 methoxy-4-vinylphenol	183.0
		10.44	vanillin	28.6
		16.78	4 hydroxy-2-methoxycinnamaldehyde	66.3
		18.41	ethyl ((2E)3-(4hydroxy-3-methoxyphenyl)-2-propenoate	273.9

Table 9. continued

Biomass	Treatment (% chemical wt/wt of dry biomass)	GC-MS Retention time (min)	Compound	Total Phenols (µg vanillin g^{-1} dry bagasse)
CLM – cont'd.	50% Ethanol &5% NaOH => 5% NaOH	8.72	2 methoxy-4-vinylphenol	4.7
		9.66	benzaldehyde, 4-hydroxy	5.2
		10.44	vanillin	23.7
		15.48	benzaldehyde, 4 hydroxy-3,5-dimethoxy	4.6
Pith	5% NaOH	8.72	2 methoxy-4-vinylphenol	39.6
		9.68	benzaldehyde, 4-hydroxy	443.2
		10.44	vanillin	177.1
		12.18	ethanone, 1-(4-hydroxy-3-methoxyphenyl)	29.5
		15.48	benzaldehyde, 4-hydroxy-3,5 dimethoxy	53.2
		16.23	3 phenylphenol	1202.8
		16.82	ethanone, 1-(4 hydroxy-3,5-dimethoxyphenyl)	42.5
	5% NaOH => 5% NaOH	9.66	benzaldehyde, 4-hydroxy	52.6
		10.44	vanillin	110.4
		15.48	benzaldehyde, 4 hydroxy-3,5-dimethoxy	93.8
		16.23	3-phenylphenol	952
	50% Ethanol & 5% NaOH	6.81	benzofuran, 2,3 dihydro	2800
		8.72	2 methoxy-4-vinylphenol	606.5
		9.69	benzaldehyde, 4-hydroxy	332.6
		10.44	vanillin	103.2
		14.13	benzoic acid, 4-hydroxy-3-methoxy, ethyl ester	13.1
		15.49	benzaldehyde, 4-hydroxy-3,5-dimethoxy	74.8
		16.55	p-hydroxycinnamic acid, ethyl ester	67.9
		16.77	4 hydroxy-2-methoxycinnamaldehyde	59.5
		16.82	ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)	70.1
		19.94	ethyl ((2E)3-(4hydroxy-3-methoxyphenyl)-2-propenoate	35.8
	50% Ethanol &5% NaOH => 5% NaOH	4.54	phenol, 2-methoxy	16.8
		8.72	2 methoxy-4-vinylphenol	78.3
		9.66	benzaldehyde, 4-hydroxy	151.9
		10.44	vanillin	212.0
		15.48	benzaldehyde, 4-hydroxy-3,5-dimethoxy	114.8
		16.81	ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)	43.4

GC analyses were conducted on extracted samples to determine vanillin concentrations from each biomass fraction. A summary of quantification efforts is given in compounds in Tables 10 - 12.

Table 10: Simple phenols from bagasse as a function of treatment.

Treatment	F.C. Phenols* mg/g dry biomass	# compounds detected	Detected % of F.C. Phenols	Vanillin % of F.C. Phenols	3 most prevalent phenols, % of F.C. Phenols
NaOH	35.5	10	48	9.7	27
NaOH/NaOH[1]	50.2	9	32.8	12.75	22.4
EtOH-NaOH[2]	29.4	12	40	8.3	20.6
EtOH/NaOH[1]	30.8	25	100	6.4	37
Ox-B /NaOH[1]	46.6	11	23.7	2.4	10.9
OxB-EtOH[2]/NaOH[1]	50.3	12	40.5	5.4	19.3

* simple phenols detected by Folin Ciocalteu Method
1. /treatment followed by a second NaOH treatment
2. combined treatments

Table 11: Simple phenols from CLM as a function of treatment.

Treatment	F.C. Phenols* mg/g dry biomass	# compounds detected	Detected % of F.C. Phenols	Vanillin % of F.C. Phenols	3 most prevalent phenols, % of F.C. Phenols
NaOH	46.9	15	42.6	7.5	26.2
NaOH/NaOH[1]	39.8	12	26.9	6.9	12.7
EtOH-NaOH[2]	N/A	N/A	N/A	N/A	N/A
EtOH-NaOH[2] /NaOH[1]	30.5	20	38.2	7.8	19.5

* simple phenols detected by Folin Ciocalteu Method
1. /treatment followed by a second NaOH treatment
2. combined treatments

Table 12: Simple phenols from pith as a function of treatment.

Treatment	F.C. Phenols* mg/g dry biomass	# compounds detected	Detected % of F.C. Phenols	Vanillin % of F.C. Phenols	3 most prevalent phenols, % of F.C. Phenols
NaOH	31.4	11	70.4	9.0	36.2
NaOH/NaOH[1]	30.8	6	24.9	7.4	16.8
EtOH-NaOH[2]	42.8	20	70.2	1.1	46.4
EtOH-NaOH[2] /NaOH[1]	30.5	8	31.9	10.4	22.4

* simple phenols detected by Folin Ciocalteu Method
1. treatment followed by a second NaOH treatment
2. combined treatment

Pretreatment of bagasse with sodium hydroxide resulted in the highest amount of vanillin extracted (Figure 5). Sugarcane bagasse yielded 8.17 mg of vanillin g^{-1} dry biomass, followed by CLM (6.26 mg of vanillin g^{-1} dry biomass) and pith (5.06 mg of vanillin g^{-1} dry biomass) (Figure 6).

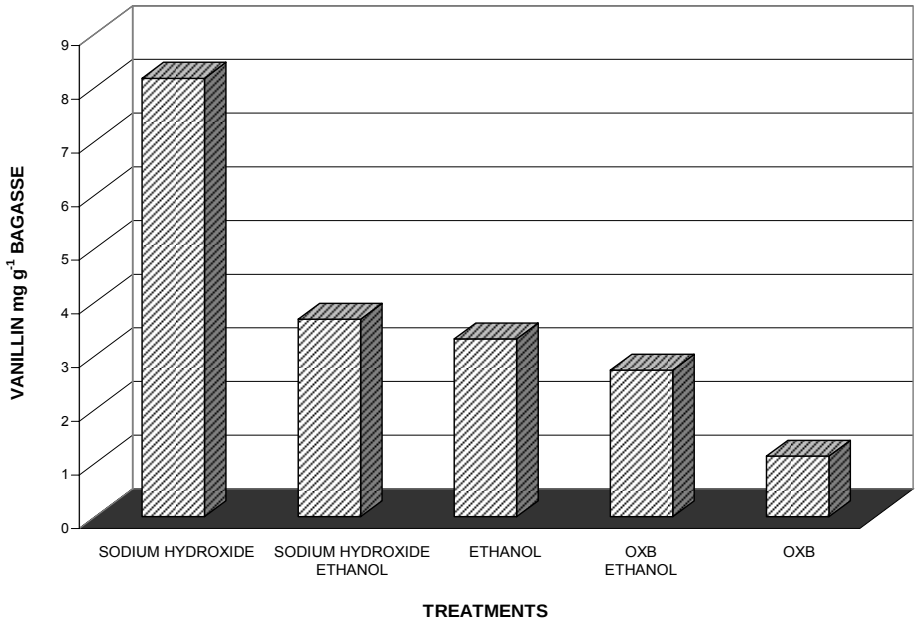


Figure 5: Vanillin Extracted from Sugarcane Bagasse as a Function of Treatment.

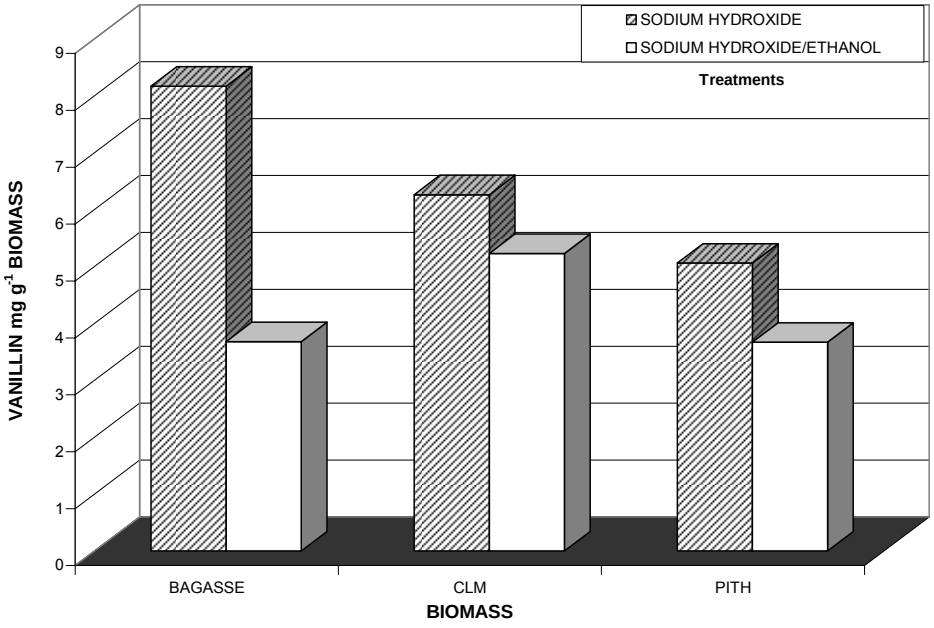


Figure 6: Vanillin Extracted from Sources of Sugarcane Biomass.

Treatments of sugarcane bagasse with sodium hydroxide, ethanol, Ox-B, and combinations thereof produced both chlorinated and non-chlorinated phenolic compounds. Some tentatively identified compounds have commercial value.

Investigatation of lignin degradation products from hypochlorite pretreatment of bagasse
Because of reports in the patent literature on the role of manganese in degrading peroxides, the effects of hydrogen peroxide and manganese sulfate combinations on lignin degradation were evaluated. It was thought that a manganese catalyst may produce bursts of radicals that would cleave polymer lignin to monophenols. Total phenolic compound values obtained on hydrogen peroxide and manganese sulfate treatments (ethanol, NaOH, Ox-B or combinations thereof pretreatments) of sugarcane bagasse extracts are presented in Table 13. TPC increased in liquors after hydrogen peroxide/manganese sulfate treatment on ethanol or Ox-B treated bagasse liquors up to 10 and 6 fold respectively. Heated peroxide/manganese treatments were less effective than room temperature treatments. A decrease in TPC was observed on samples treated with heat and peroxide/manganese. It is possible that sodium hydroxide treated bagasse released monophenols and lignin which when treated with the oxidant hydrogen peroxide/manganese sulfate solution were oxidized to compounds not detected as TPC. Hydrogen peroxide/manganese sulfate combination generates hydroxyl radicals which are known to oxidize phenols.

Increased concentrations of hydrogen peroxide/manganese sulfate solution produced little increase in TPC values for ethanol and Ox-B treated samples. No significant increase in the amount of total phenols released was observed on heating treated sugarcane bagasse extracted liquors with hydrogen peroxide/manganese sulfate solutions. No significant differences were observed when hydrogen peroxide/manganese sulfate solutions were added as mixtures or as individual compounds to each sample.

Table 13: Total phenolic compounds (TPC) before and after treatment of extract liquors obtained from treated sugarcane bagasse with hydrogen peroxide and manganese sulfate.

Sequential Treatments % chemical/dry wt. bagasse	Total Phenols (mg/g) as Vanillin equivalents								
	Before	Hydrogen Peroxide/Manganese Sulfate							
		Room Temperature				Heated*			
		# 1%	#0.5%	^ 1%	^0.5%	#1%	#0.5%	^1%	^ 0.5%
5% NaOH/ 12,500% (w/w) ETOH	34.3	27.7	21.3	23.8	24.5	42.0	38.8	44.1	41.2
Stage 1 →250% (w/w) ETOH	5.0	9.4	4.0	9.1	5.5	2.9	2.5	2.4	2.0
Stage 1 → 5% NaOH	31.9	20.6	21.9	20.6	20.4	29.0	21.0	17.7	22.5
12,500% (w/w) ETOH	5.1	18.1	9.7	24.0	17.3	43.6	36.1	43.6	34.3
Stage 1 →250% (w/w) ETOH	1.4	15.9	4.6	10.3	13.1	14.1	10.2	10.2	14.0
Stage 1 → 5% NaOH	38.2	19.7	19.4	16.5	29.9	15.1	14.8	14.6	14.3
5% NaOH	33.8	25.4	25.0	23.7	23.2	22.2	22.8	19.7	21.8
Stage 1 → 250% (w/w) EtOH	13.4	12.1	8.8	16.3	11.3	7.3	5.1	8.0	7.6
Stage 1 →5% NaOH	44.6	35.6	36.4	32.9	32.4	36.6	39.2	34.2	35.2
10% Ox-B	4.0	12.4	10.0	15.3	11.9	9.8	10.0	9.7	9.1
Stage 1 → 250% (w/w) ETOH	2.1	7.2	6.0	11.7	11.4	10.6	8.4	13.0	11.2
Stage 1 → 5% NaOH	43.9	29.3	30.8	27.6	29.3	29.5	29.5	30.5	33.4

*= Samples heated at 90°C for 1 h.
#= Hydrogen peroxide and manganese sulfate were each added individually (I) to each sample.
^ = Hydrogen peroxide and manganese sulfate were mixed (M) and added to each sample.

Lignin extracts from ethanol or Ox-B treated bagasse, unlike NaOH treated extracts produced high quantities of monophenols when treated with peroxide/manganese solutions.

Cellulose hydrolysis

The conditions used for simultaneous saccharification/ fermentation at a pilot level for the production of ethanol are classed as cellulose hydrolysis I while cellulose hydrolysis II are those conditions which yielded the highest concentrations of cellobiose. Addition of glucose oxidase to a celluloytic saccharification system shifted the reaction equilibria toward cellobiose. A recently obtained transglycosylase has the ability to add xylose, fructose, galactose and other monosaccharides to cellobiose. This opens up the possibility of producing a range of oligomers, with as yet unknown properties, from cellulobiose.

The commercial cellulase enzyme, synthesized by a *Trichoderma viride* strain, is a mixture of cellulase component such as endo- and exo-glucanases and b-glucosidase. All enzyme activities were assessed using the same enzyme, the *T. viride* cellulase complex, but distinguished cellulase activity was distinguished by substrate. Phosphoric acid swollen Avicel was used as the primary substrate for

cellulase and the b-glucosidase activity was determined against cellobiose. The hydrolysis rates for kinetic studies were determined after 30 min of hydrolysis to minimize the inhibitory effects of the other released sugars. The degree of production of cellobiose was compared after 24 h of hydrolysis because rapid release of glucose and cellobiose was observed in the first 24 h of hydrolysis, and 24 h is the prescribed time of saccharification in a SSF process.

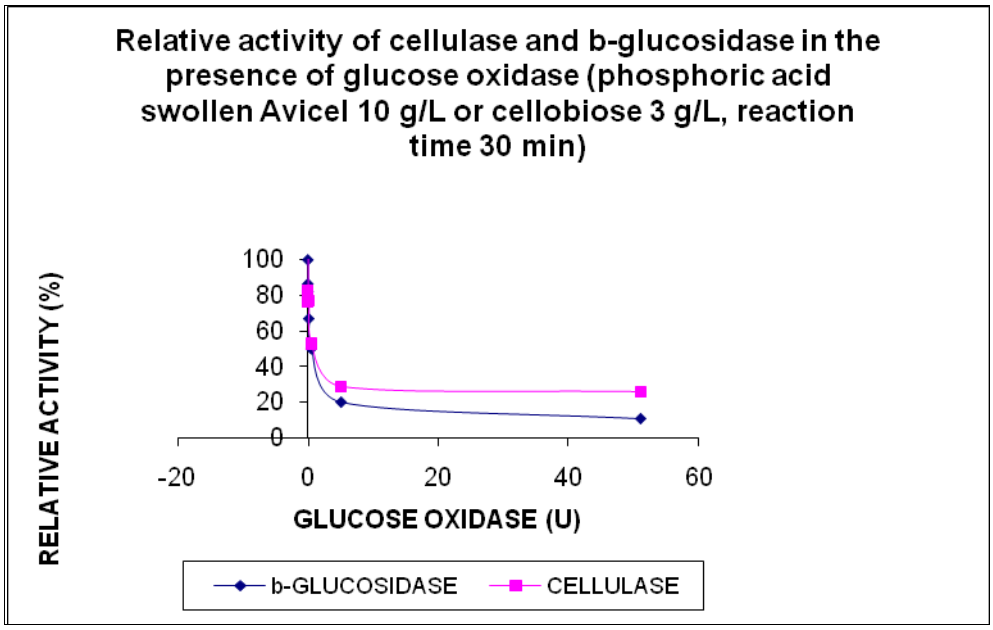


Figure 7. Effect of glucose oxidase loading on apparent activity of cellulases and b-glucosidases.

A glucose oxidase loading over the range of 0 to 51 U was tested for its effect on the individual activities of cellulase and b-glucosidase. Cellulose at 10 g/L was used as substrate and cellobiose at 3 g/L for b-glucosidase. No differences were observed between the activities of these enzymes by the presence of glucose oxidase. At concentrations higher than 5.1 U of glucose oxidase, the cellulase lost 71% and b-glucosidase 80% of their activity (Figure 7).

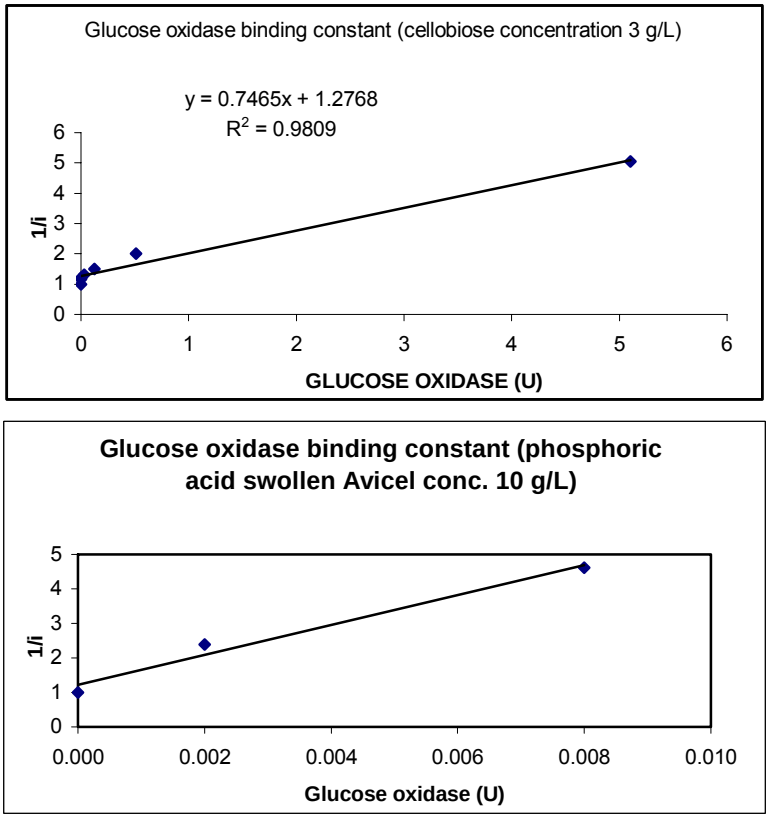


Figure 8. Apparent inhibitor binding constants for glucose oxidase against Avicel and cellobiose.

Figure 8 gives the apparent inhibition constant for glucose oxidase. Note that glucose oxidase not actually bind to the enzyme(s) but oxidizes glucose.

Hydrolysis was performed at 50C for 38 h with 10 g/L of phosphoric acid swollen Avicel with various levels of glucose oxidase (Figure 9). The yield of cellobiose increased with increased glucose oxidase loading. Significant increase in cellobiose concentrations were observed over a glucose oxidase concentration above 0.51 U. Highest cellobiose yield was 19.3% after 24 h of hydrolysis with 51 U of glucose oxidase, whereas it was only 2.4% without any glucose oxidase addition. A rapid decrease was observed after reaching a plateau in samples with glucose oxidase.

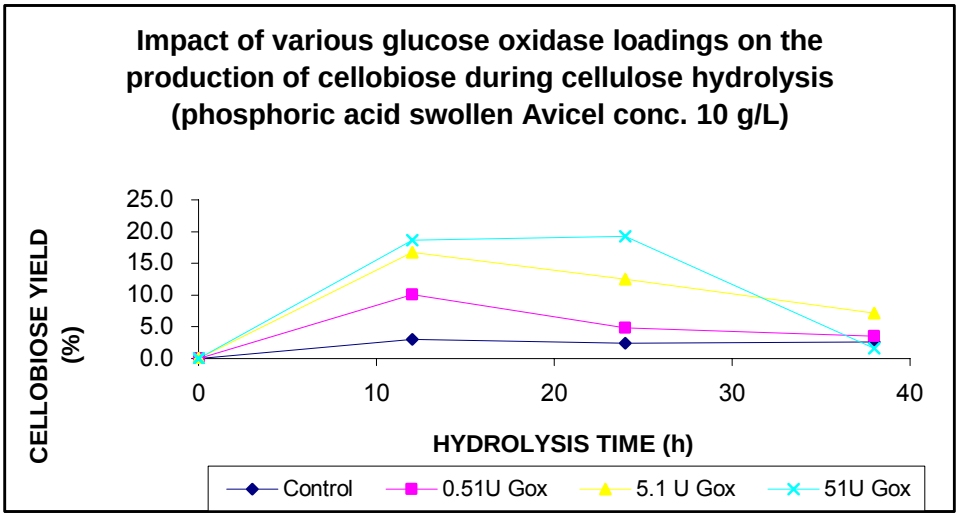


Figure 9. Impact of glucose oxidase loading on cellobiose production

Role of Gluconolactone

Because the product of glucose oxidase from glucose is gluconolactone, it was tried as an inhibitor of cellobiose conversion to glucose. This was to determine the mechanism of action rather than as a practical method, as the cost of gluconolactone is significantly higher than that of glucose oxidase. Figure 10 shows the effect of gluconolactone on the relative activity of cellulase and b-glucosidase.

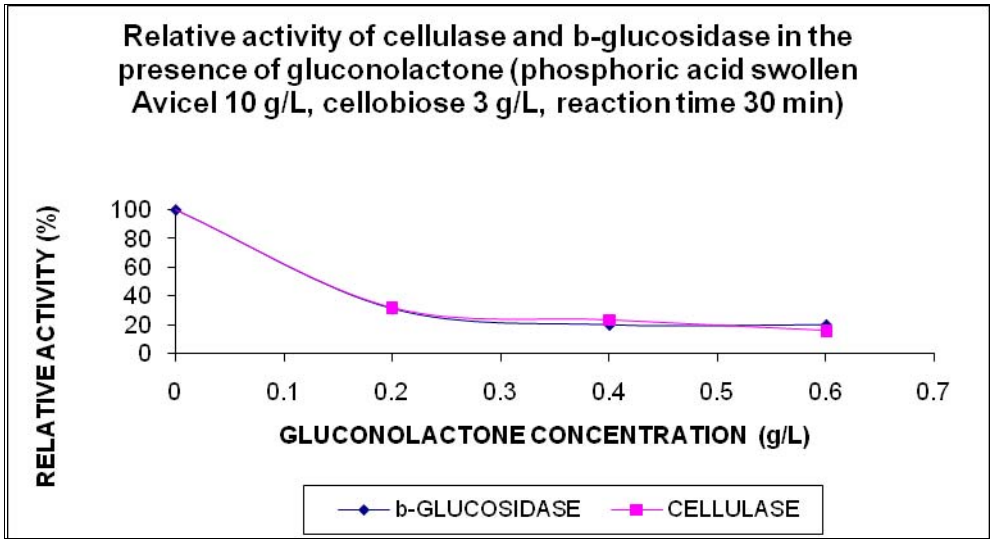


Figure 10. Effect of gluconolactone of cellulase and b-glucosidase activity.

Gluconolactone showed same degree of inhibition on the activities of cellulase and b-glucose oxidase (data not shown). The gluconolactone inhibited selectively b-glucosidase. There is only 30% of b-glucosidase activity remaining at a gluconolactone concentration of 0.2 g/L with steady decrease with increasing gluconlactone concentrations.

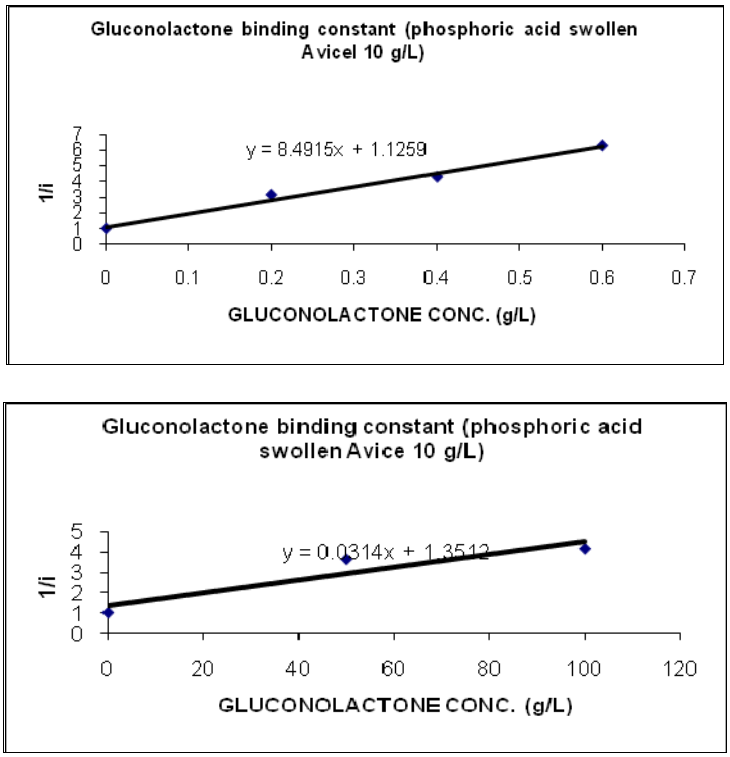


Figure 11. The binding constant of gluconolactone to Avicel

Figure 11 shows a binding constant plot (1/*i* vs. gluconolactone concentration) where the inhibition parameter *i* for a single inhibitor is calculated using the ratio of the hydrolysis rates without and with the presence of gluconolactone, $V_{\text{no inhibitor}}/V_{\text{with inhibitor}}$. The data are linear with an intercept of about 1.0 and different slopes on two ranges of gluconolactone. Linear regression ranged from 0 to 0.6 g/L of gluconolactone, generating a slope of 8.4915 and from 0 to 100 g/L a slope of 0.0314. The type of inhibition generated by gluconolactone is classed as mixed inhibition as the lines intersect below the axis. The plot is shown in Figure 12.

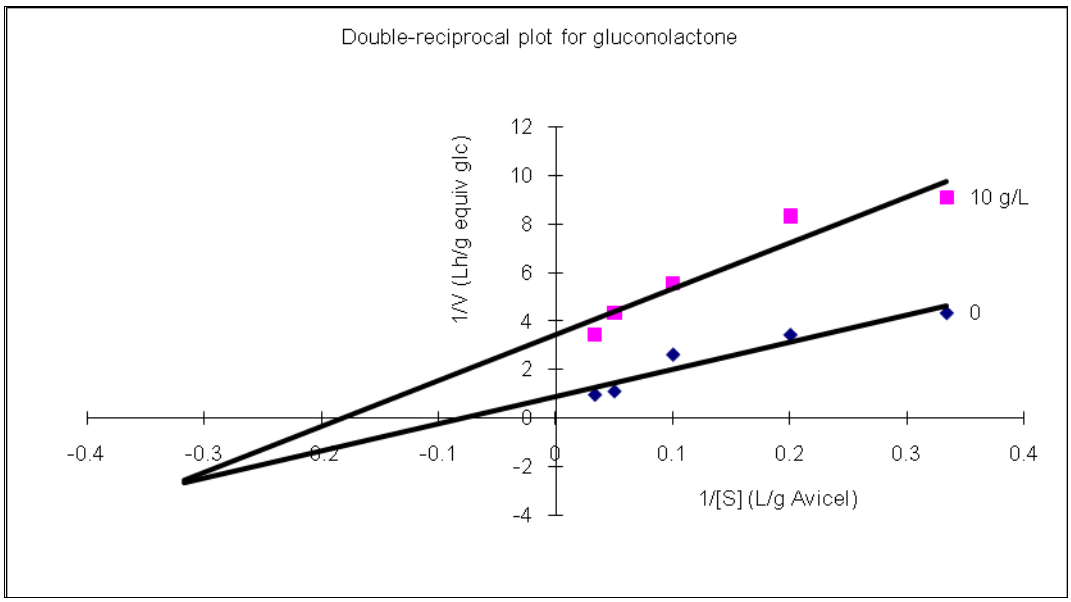


Figure 12. Double –reciprocal plot for inhibition by gluconolactone.

When a range of gluconolactone concentrations were added to reaction mixtures of 10 g/L of Avicel and cellulase, there were significant differences in the quantity of cellobiose produced over 38 h of hydrolysis. The highest yield of cellobiose, 30.6% of theoretical, was achieved at 10 g/L gluconolactone supplementation, after 24 h of hydrolysis. Excess gluconolactone addition caused inhibition of both cellobiose release as well as glucose production (Figure 13).

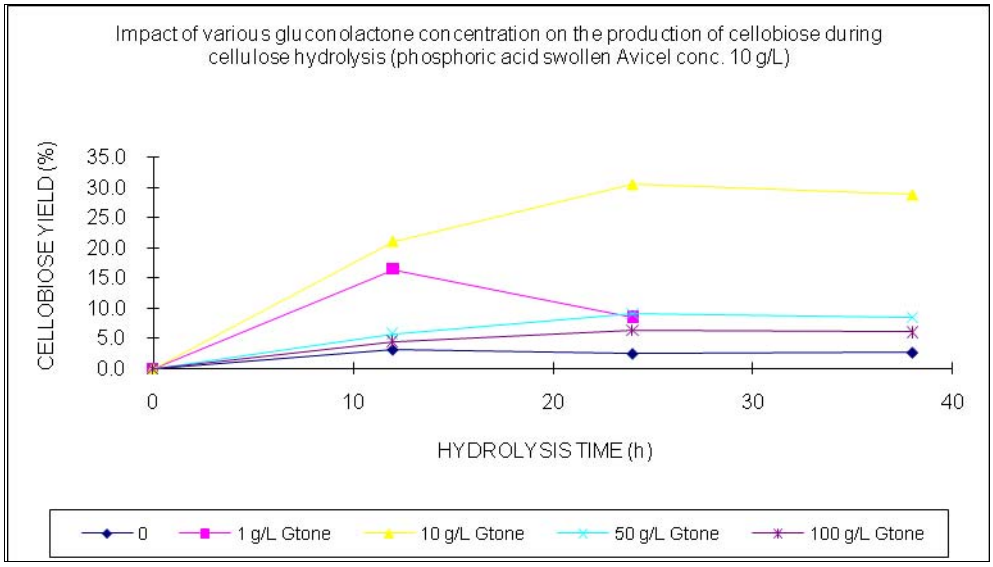


Figure 13. Impact of gluconolactone concentration on cellobiose production.

Role of gluconic acid

The effect of gluconic acid concentrations from 0 to 50 g/L on the cellulase and b-glucosidase activities were determined (Figure 14). At concentration of 10 g/L cellulase, there was a 78% decrease in cellulase activity and a 90% decrease in b-glucosidase activity.

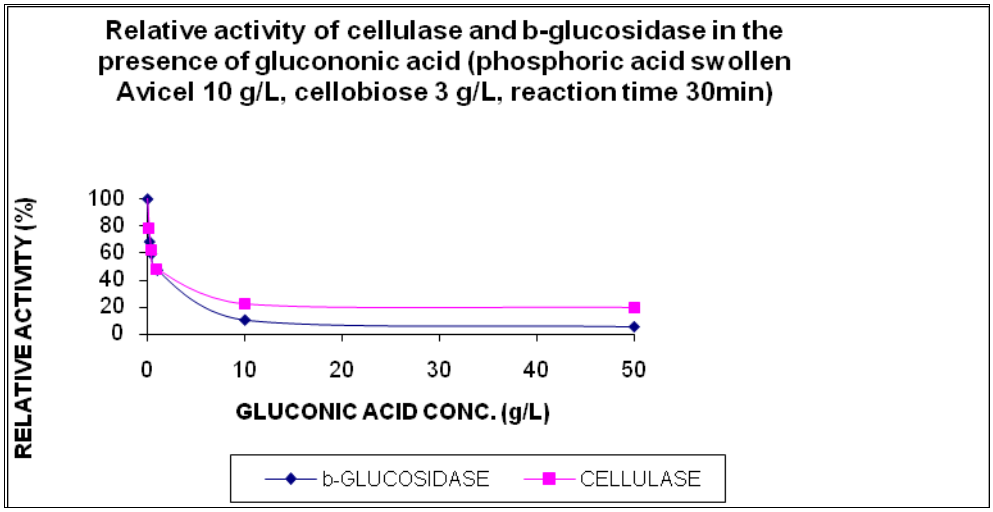


Figure 14. Effect of gluconic acid addition on cellulase and b-glucosidase activities.

Figure 15 shows a binding constant plot (1/*i* vs. gluconic acid concentration) for gluconic acid to Avicel. The slope has a value of 1.0582 L/g at a gluconic acid concentration from 0 to 0.7 g/L and 0.046 L/g at gluconic acid concentration ranged from 0 to 100 g/L.

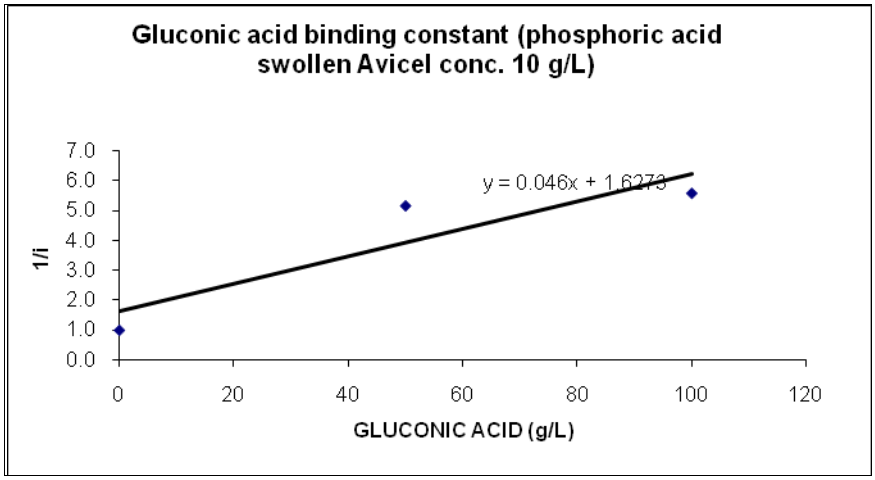


Figure 15. Binding constant of gluconic acid to Avicel.

Gluconic acid exhibited mixed inhibition since the line intersects in the lower left quadrant of a double reciprocal plot (Figure 16).

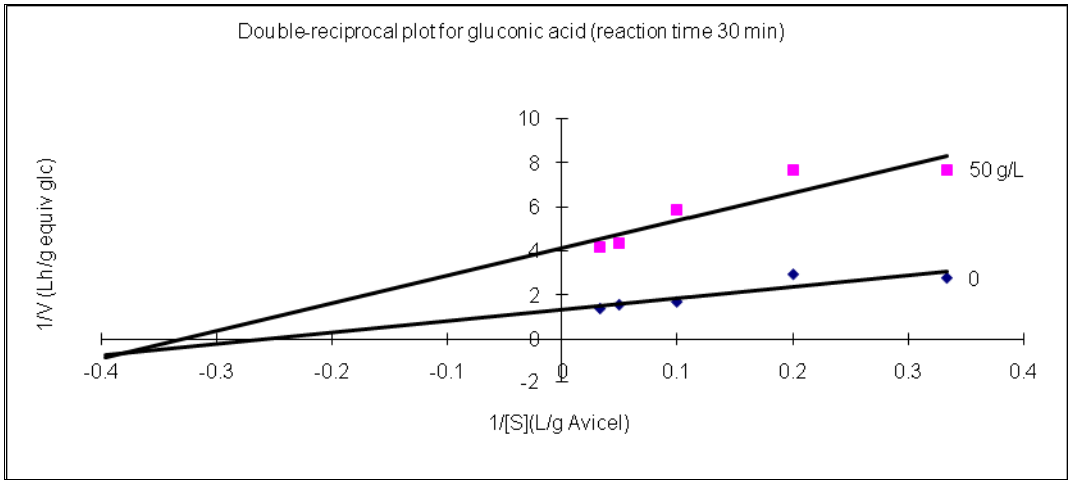


Figure 16. Double reciprocal plot for gluconic acid inhibition.

Gluconolactone supplementation significantly increased cellobiose concentrations in the reaction mixture on cellulose hydrolysis. The addition of 50 g/L and 100 g/L gluconic acid showed similar patterns in cellobiose yield (Figure 17). The highest yield of cellobiose was 29.7% of theoretical with 50 g/L gluconic acid supplementation at 38 h hydrolysis. It was ten times higher than the control sample (without gluconic acid).

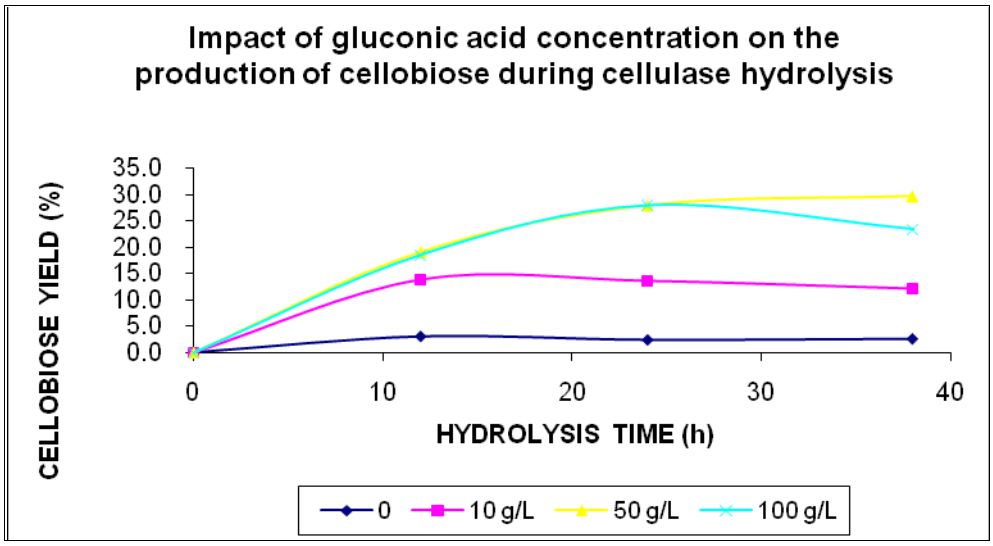


Figure 17. Impact of gluconic acid on cellobiose yields.

Cellobiose production from lime pretreated sugarcane bagasse

Dried lime pretreated sugarcane bagasse after swelling in a buffer overnight at 50 °C was hydrolyzed similar to Avicel. However, it needed longer incubation time to reach the maximum hydrolysis rate than moist pretreated bagasse or phosphoric acid swollen Avicel (Figure 18).

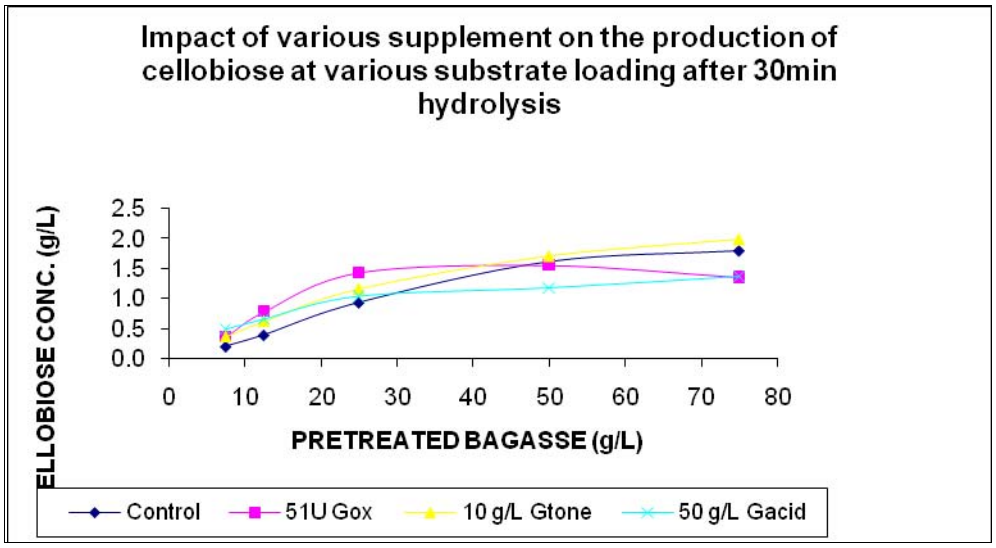


Figure 18. Impact of supplements on cellobiose production from pre-treated bagasse

After 24 hr of hydrolysis cellobiose production approached the same level for all supplements (Figure 19).

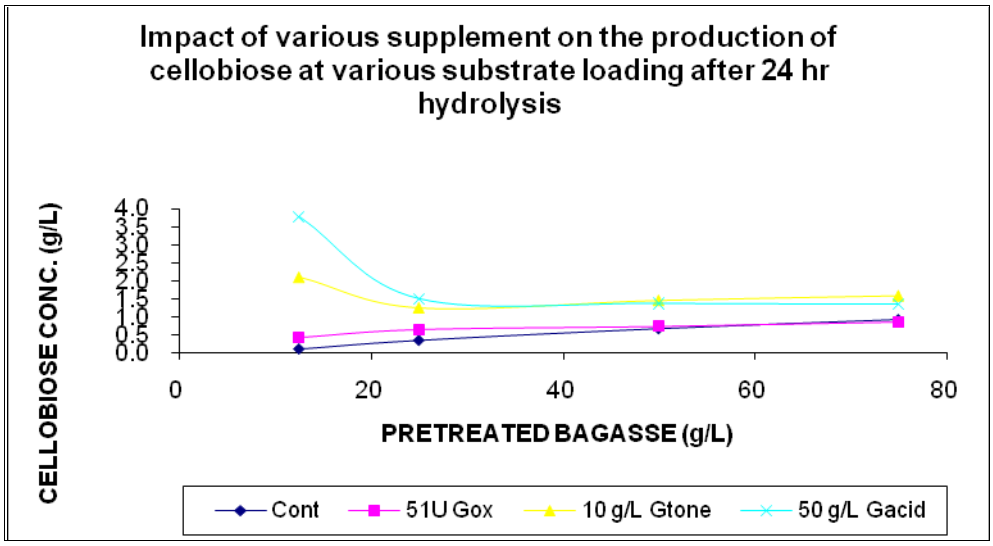


Figure 19. Impact of various supplements on cellobiose production after 24 hr from pretreated sugarcane bagasse.

The order of cellobiose enhancement was, for 12.5 g/L of pretreated bagasse, gluconic acid at 50 g/L produced 3.8 g/L cellobiose, followed by 10 g/L gluconolactone (2.1 g/L cellobiose), 51 U glucose oxidase (0.4 g/L cellobiose), and then the control sample, without any supplement, gave 0.1 g/L cellobiose (Figure 20). Increasing the amount of substrate decreased impact of each supplements on the production of cellobiose.

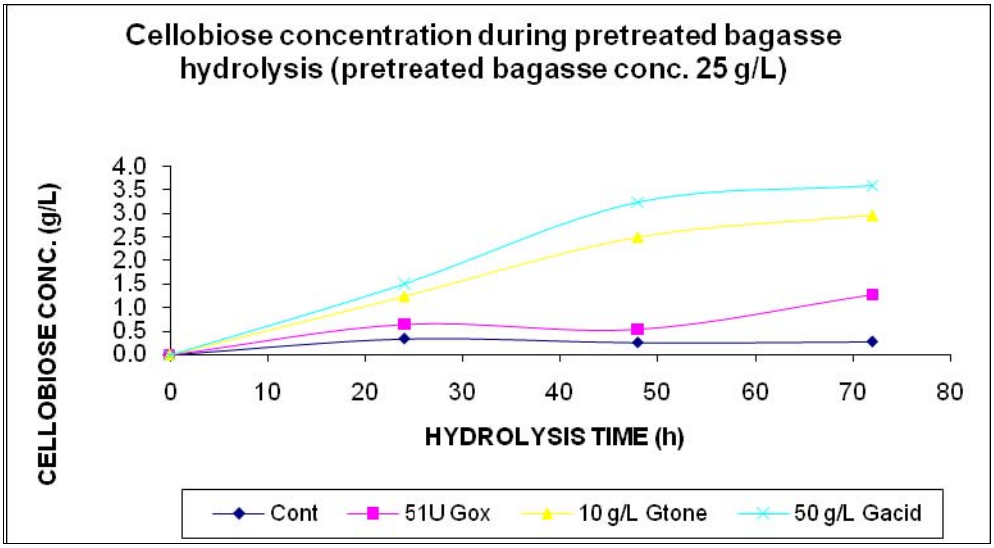


Figure 20. Cellobiose production by addition of individual b-glucosidase inhibitors to a sugarcane bagasse hydrolysis reaction.

Cellobiose concentration increased until 24 hr in a control sample (without any supplement) and then plateaued. However, addition of the other supplements, such as 51 U glucose oxidase, 10 g/L gluconolactone, or 50 g/L gluconic acid produced a steady and significant increase in cellobiose with time. After 72 hr of hydrolysis the addition of 50 g/L gluconic acid produced 3.6 g/L cellobiose, followed by 10 g/L gluconolactone (3.0 g/L cellobiose), 51 U glucose oxidase (1.3 g/L cellobiose, and no supplement (0.3 g/L cellobiose).

Cellobiooligosaccharides

Cellobiose was used as a receptor for transglycosylation reactions to produce a range of novel oligosaccharides (CBO) which have some unique properties. Details of the production are under patent consideration and are not disclosed here.

Cariogenicity - The CBO inhibited effectively the synthesis of water insoluble glucans in the presence of sucrose by mutansucrase (Figure 21). In the presence of 50 mM CBO and 1 M sucrose, only 4% of insoluble glucans were produced comparing to that of a 1 M sucrose reaction mixture. Cellobiose (50 mM) did not affect insoluble glucan formation as almost same amount of insoluble glucans was produced as in the control. Insoluble glucans in solution were swirled along the inner layer of a glass vial, and the liquid discarded. Then the carbohydrates were dyed with a dental disclosing solution. The insoluble glucans adhered as an inner layer on a glass vial as dental plaque does on teeth (Figure 22). The quantity of insoluble glucans adhered to a glass was less for the CBO mixture than the control of cellobiose test. The inhibitory effect of CBO against mutansucrase might be caused by an acceptor reaction of glucosyltransferase, leading to termination of glucan synthesis from sucrose. Our data are consistent to that observed with isomaltosylfructoside (Nisizawa et al., 1986), fructosylxyloside (Takeda and Kinosh, 1995), maltosylsucrose (Lee et al., 2003), and glucooligosaccharides and fructooligosaccharides (Nam et al., 2007), Dental caries are mostly caused by *S. mutans* and *S. sobrinus* which synthesize extracellular water insoluble glucans from sucrose by glucosyl transferase (Hamada and Slade, 1980). The insoluble glucans become plaque on teeth and result in tooth decay. In addition, *S. mutans* and *S. sobrinus* synthesize intracellular polysaccharides as carbohydrate reserves, which can be converted to acids when dietary carbohydrates are available. CBO has a potential to become an active ingredient in dental care products since it inhibits glucan formation.

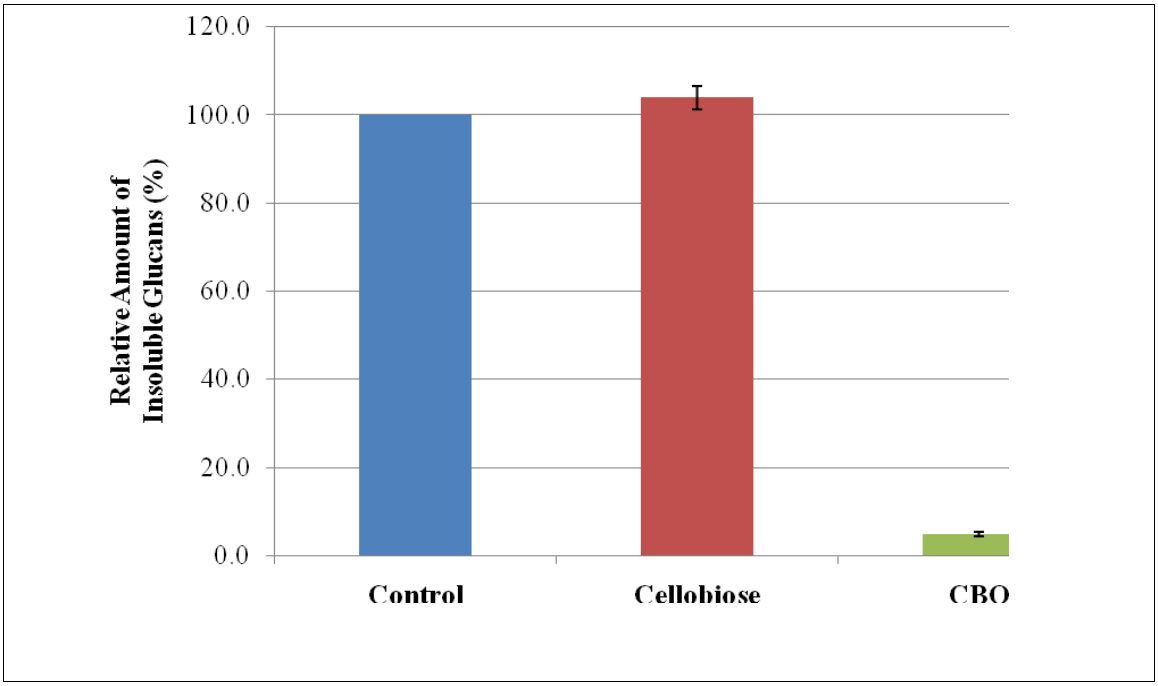
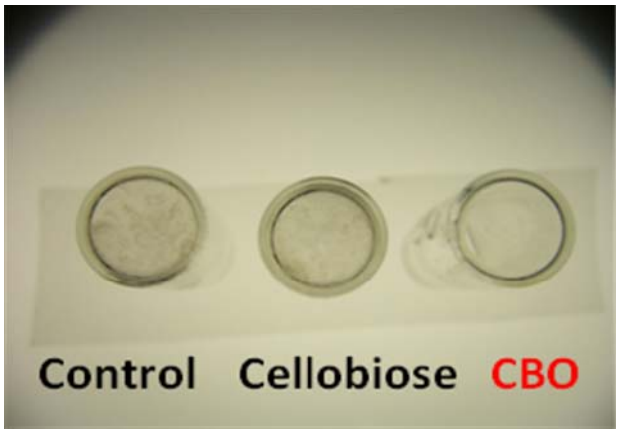


Figure 21. Effect of addition of cellobiose or CBO on the formation of insoluble glucan by mutansucrase**.** Relative amount of insoluble glucans is the percent of the amount of insoluble glucans in the test to that in the control mixture. Error bars show the standard deviation errors of the mean.

A.



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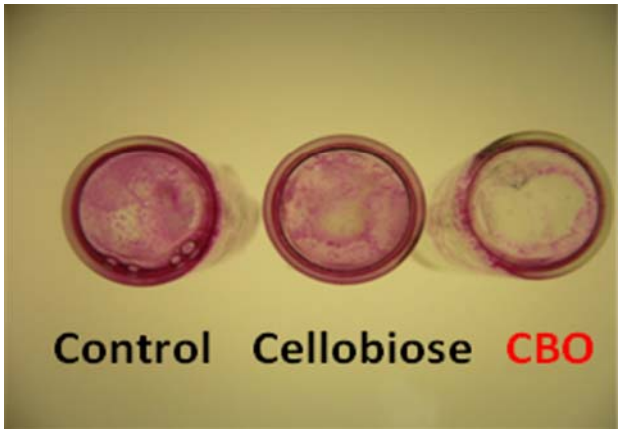

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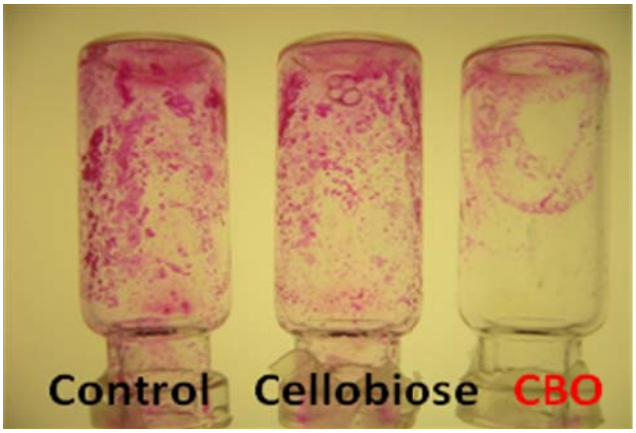


Figure 22. Insoluble glucans synthesized under different conditions in glass vials. Panel A, before addition of disclosing solutions; Panel B and C, after addition of disclosing solutions. Insoluble glucans were synthesized for 48 hours at 37 °C by mutansucrase in the presence of 1 M sucrose and 20 mM HEPES (for a control), 50 mM cellobiose (for a cellobiose), or 50 mM CBO (for a CBO).

α-Glucosidase Inhibition - The inhibitory activity of CBO was against α-glucosidase (Figure 23). Increasing concentrations of CBO decreased the amount of glucose produced from maltose by α-glucosidase. α-Glucosidase activity was 86% of control in the presence of 0.7 mM CBO, 77% in the presence of 2.0 mM CBO, and 55% in the presence of 3.3 mM CBO. Inhibition of α-glucosidase can delay carbohydrate digestion and glucose absorption, attenuating postprandial hyperglycemia (Bischoff, 1995; Lee, 2005). At present, α-glucosidase inhibitors such as acarbose, miglitol, and voglibose are commonly used to reduce the postprandial hyperglycemia by interfering with the digestion of dietary carbohydrates (Moordian and Thurman, 1999; Williamson et al., 1992). Acarbose is widely used as a therapeuctic agent for the treatment of patients with type II diabetes mellitus (Chiasson et al., 2002; Balfour, 1993). Concern about its side effects is driving the search for alternative safe and efficient α-glucosidase inhibitors.

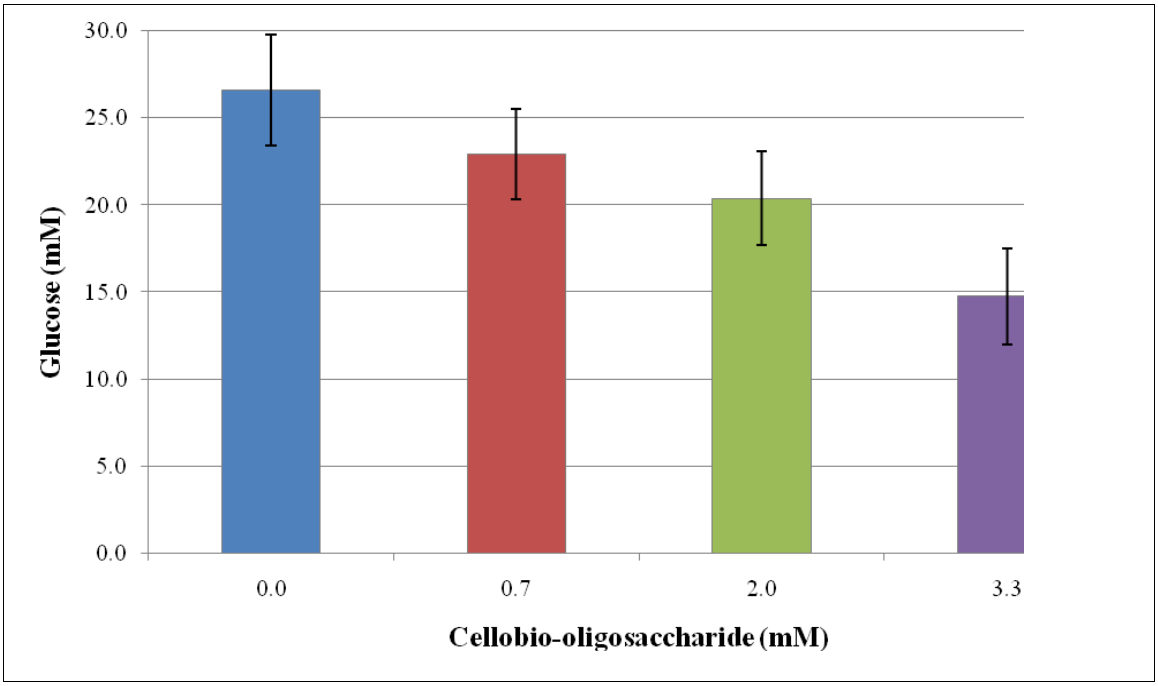


Figure 23. Inhibitory effect of CBO against α-glucosidase**.** The reaction mixture contained 4.9 U α-glucosidase, 88 mM maltose, and different concentration of cellobio-oligosaccharides (0, 0.7, 2.0, and 3.3 mM) in 20 mM HEPES buffer and incubated for 90 min at 25˚C. Error bars show the standard deviation errors of the mean.

Antifungal Effect.-Inhibition of Glucan Synthase - Antifungal agents, the GS inhibitors have been validated as an effective treatment of fungal infections because these agents inhibit fungal cell wall synthesis, a target unique to lower eukaryotes (Onishi et al, 2000). The effect of CBO on GS, the essential enzyme that forms β-(1,3)-glucan fibrils from UDP-glucose, was evaluated. Inhibition was largely dose dependent (Figure 24). The concentration of 0.12 g/ml CBO was not sufficient to inhibit GS activity. The 50% inhibitory concentration (IC_{50}) for CBO was 0.36 g/ml.

The role of CBO on GS was further evaluated by a kinetic study over a range of concentrations of UDP-glucose ranging from 0.05 to 8 mM with CBO added at concentrations of 0, 0.24, and 0.36 g/ml. The reaction velocity was calculated, and the Lineweaver-Burk plot of 1/[substrate] and 1/velocity at three oligosaccharide concentrations are illustrated in Figure 25. The non-parallel lines which converge at $x<0$ and $y>0$ are consistent with mixed type of inhibition.

There are no reports on the relationship of oligosaccharides containing mixed β(1→4), α(1→2), and α(1→6) linkages and fungal glucan synthases. A cellotriose, comprising glucose linked with only β-1,4, enhanced glucan synthase isolated from *Euglena gracilis* (Marechal and Goldemberg, 1964). Differences between our CBO and this cellotriose (Marechal and Goldemberg, 1964) demonstrate that the type of linkages may be important in altering glucan synthase activity. Cellobiose has been reported to be a stimulator for glucan synthase production in sugar beet (Morrow and Lucas, 1986) and *Euglena gracilis* (Marechal and Goldemberg, 1964). However stimulation by cellobiose does not stimulate the glucan synthase of *S. cerevisiae* (Lopez-Romero and Ruiz-Herrera, 1978) or of the germinating peanut, *Arachis hypogaea* (Kamat et al., 1992). A very simple sugar based chemical, δ-gluconolactone was an effective inhibitor of (1→3)-β-$_D$-glucan synthase in the sugar beet and in *S. cerevisiae* (Lopez-Romero and Ruiz- Herrera, 1978).

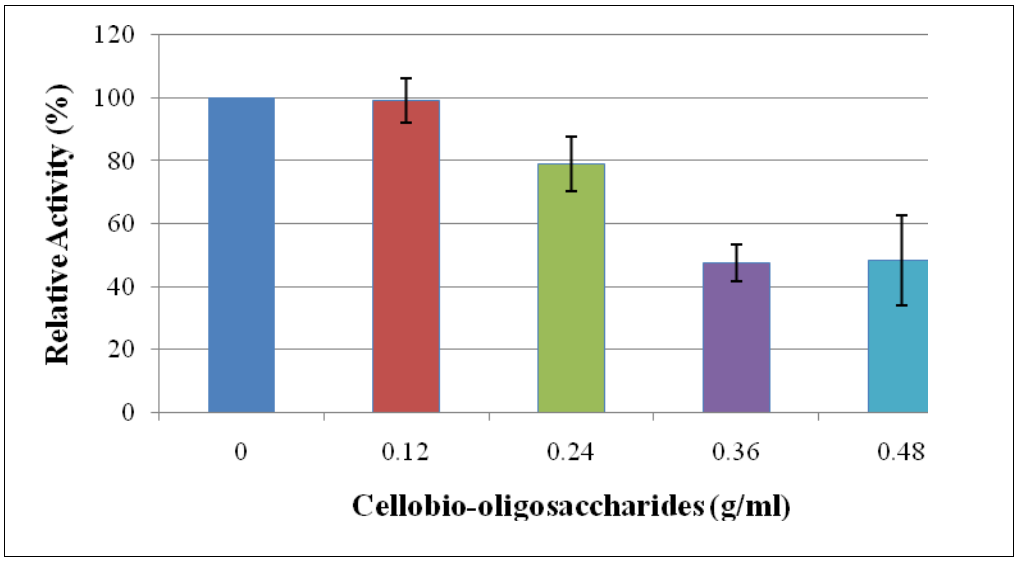


Figure 24. Inhibition of glucan synthase activity by cellobio-oligosaccharides. Relative activity is the percent of 1,3-β-$_D$-glucan synthase acitivity (GS) at test concentration of cellobio-oligosaccharides (CBO) to the GS activity at none CBO added reaction mixture (control).

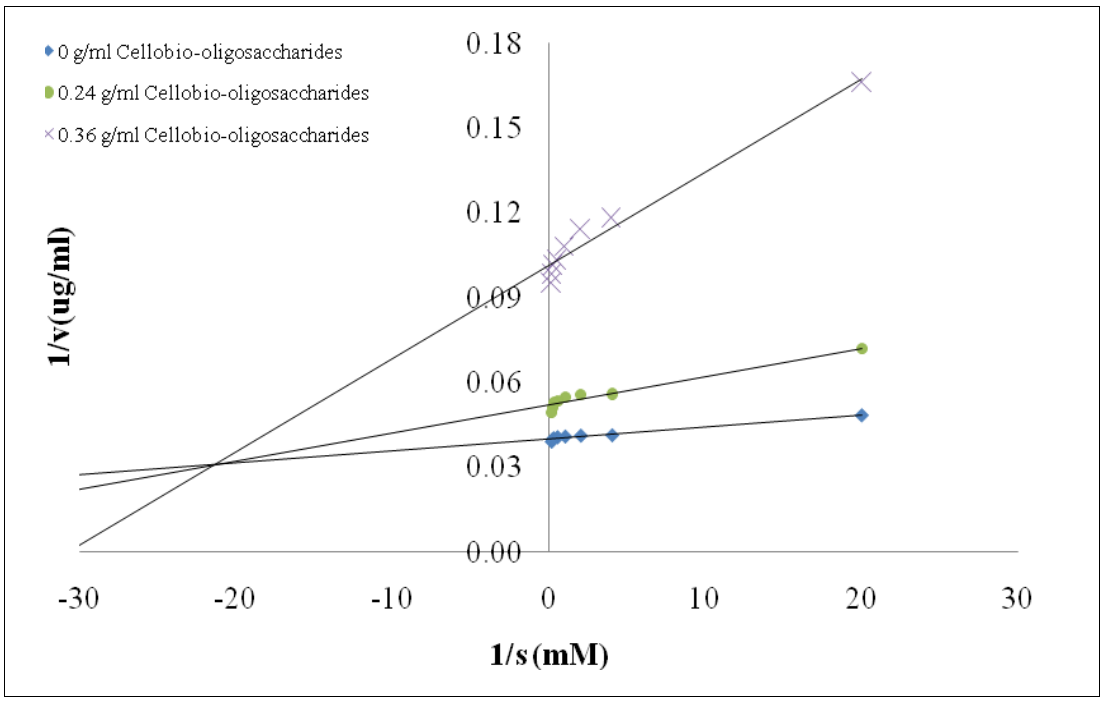
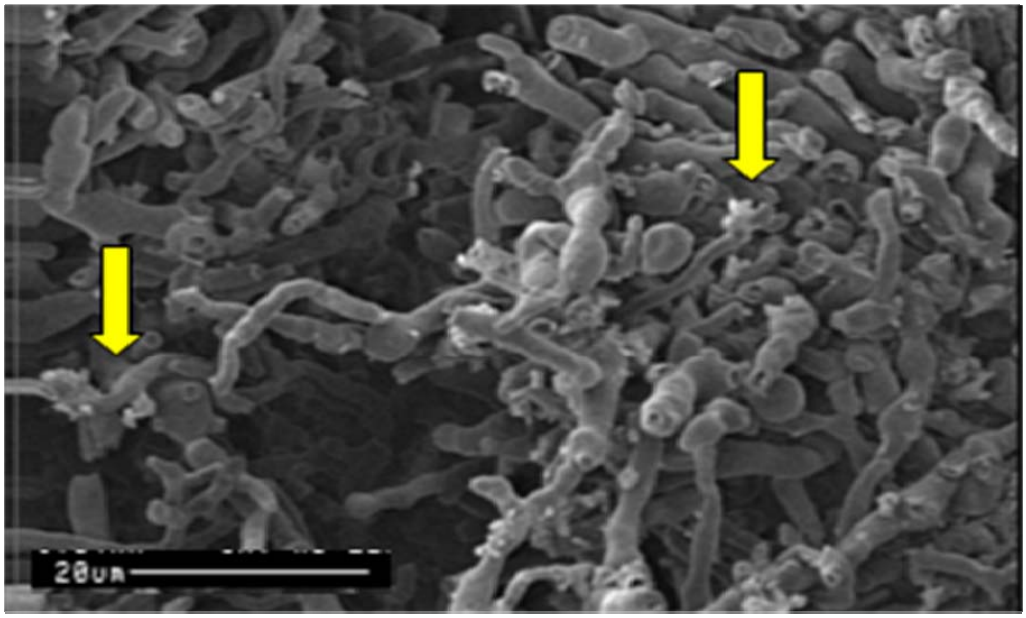


Figure 25. Lineweaver-Burk plot: inhibition of (1→3)-β-$_D$-glucan synthase by cellobio-oligosaccharide. The assay mixtures contained 27 mM HEPES (pH 7.2), 7 μM GTP, 1.3 mM EDTA, 0.17% Brij 35, and 2.2% glycerol, varying concentration of UDP-G (0.05, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 μg/μl) also containing 0.83 μg/μl 1,3-β-$_D$-glucan synthase (GS). The reaction allowed to react for 105 min at 22°C. GS activity was also measured in the absence or presence of 0, 0.24, and 0.36 g/ml cellobio-oligosaccharides. A Sirofluor™ binding with 1,3-β-$_D$-glucans was then conducted as described in the material and method section. The fluorescence was measured excitation wavelength of 390 nm and emission wavelength of 455 nm.

Lineweaver-Burk plot indicates that CBO acts as an inhibitor of glucan synthase activity. Most glucan synthase inhibitors induce profound morphological changes in fungal hyphae which correlate with inhibition of glucan synthase (Kurtz et al., 1994; Bozzola et al., 1984; Cassone et al., 1981). Observation of hyphal changes after addition of CBO was conducted using SEM. Hyphae of *A. terreus* showed distinct structural differences between control and CBO treated cultures (Figure 26). The bud scar rings are found in several hyphal tips on the control but none on CBO treated *A. terreus* in the hyphae of CBO treated *A. terreus* fail to bud their cells, and the population does not increase. In addition, ther widths of hyphae were different between two cultures when 20 hyphae were randomly selected and measured. The average width (3.4 μm) of twenty hyphae in CBO treated *A. terreus* was 1.35 fold larger than that (2.5 μm) in the control. In the presence of CBO, the cells grew with swollen hyphae, indicating inhibition of glucan synthesis (Kurtz et al., 1994). This observation supports the fact that glucan synthesis inhibition produces stunted, swollen hyphae, caused from a weakened cell wall that expands under high internal pressure (Onishi et al., 2000). In the work of Kurtz et al. (1994) pneumocandins treated *A. fumigates* caused swelling and distension of the hyphae.

Although the abundance of 1,3-β-$_D$-glucans in the cell walls formed during different stages of the *A. fumigatus* life cycle is not well characterized, the focus of new cell wall synthesis is the hyphae during vegetative growth (Archer, 1977; Beauvais et al., 2001: Ruiz-Herrera, 1992), and inhibition of 1,3-b-D-glucan synthesis has profound effects on cell wall structure in *A. fumigatus* (Kurtz et al., 1994). Inhibition of glucan synthesis results in structural changes, characterized as pseudohyphae, swollen hyphae, thickened cell wall, or buds failing to separate from mother cells (Kurtz et al., 1994; Bozzola et al., 1984; Cassone et al., 1981). We explored the effect of CBO on *A. terreus*, grown in PDB and PDA during extended incubation up to ten days at 28˚C (Figure 30). When *A. terreus* was grown in PDB medium, it formed tangled hyphal masses on the surface in a tube. However, they were not observed when *A. terreus* was incubated with CBO in PDB medium. There was substantial growth in the untreated culture during the course of the experiment.

There is no comparable data that oligosaccharide alone works as an antifungal. Almost all proposed antifungal agents have complicated structures. Nevertheless, Kaur et al. (2006) suggested that a small and simple sugar acid, $_D$-gluconic acid from *Pseudomonas* strain AN5 may have antifungal activites against take-all disease of weheat caused by *Gaeumannomyces graminis* var. *tritici*. Some researchers have been reported that cellobiose-based lipids have fungicidal activities. Complex cellobiose-lipids of yeast fungi *Cryptococcus humicola* and *Pseudozyma fusiformata* (ustilagic acid B) inhibited the growth of a number of species important for medicine:*Candida. albicans, C. glabrata, C. viswanathii, F. neoformans,* and *Clavispora lusitaninae* (Kulakovskaya et al., 2007; Kulakovskaya et al., 2006). They may stimulate the release of ATP from the test culture cells, indicating to increase the permeability of plasma membrane, and resulting in cell death (Puchkov et al., 2001; Kulakovskaya et al., 2004). Based on our data, CBO has a great potential to function as a new class of antifungal agent against fungi which correlate with 1,3-β-$_D$-glucan synthase inhibition.

A. 

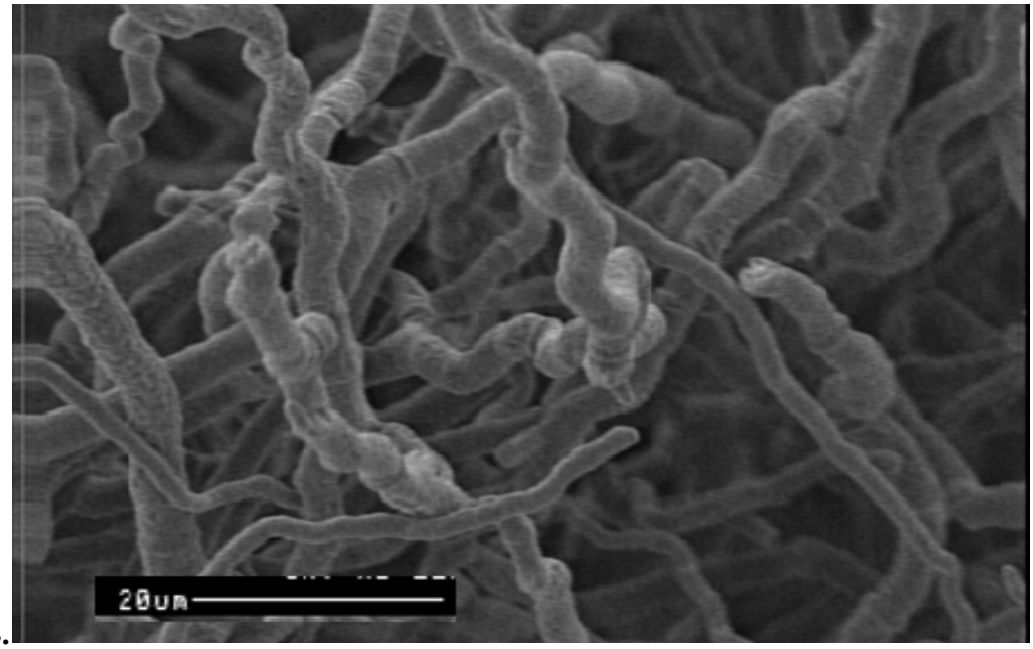
B. 

Figure 26. Scanning Electron Microscopy (SEM) images of *A. terreus* cells. Panels: A, control growth of cells (no cellobio-oligosaccharide); B, growth of cells treated with cellobio-oligosaccharides. Arrows point at sporulation of the cells. Bars represent 20 μm at 1.34 kX magnification.

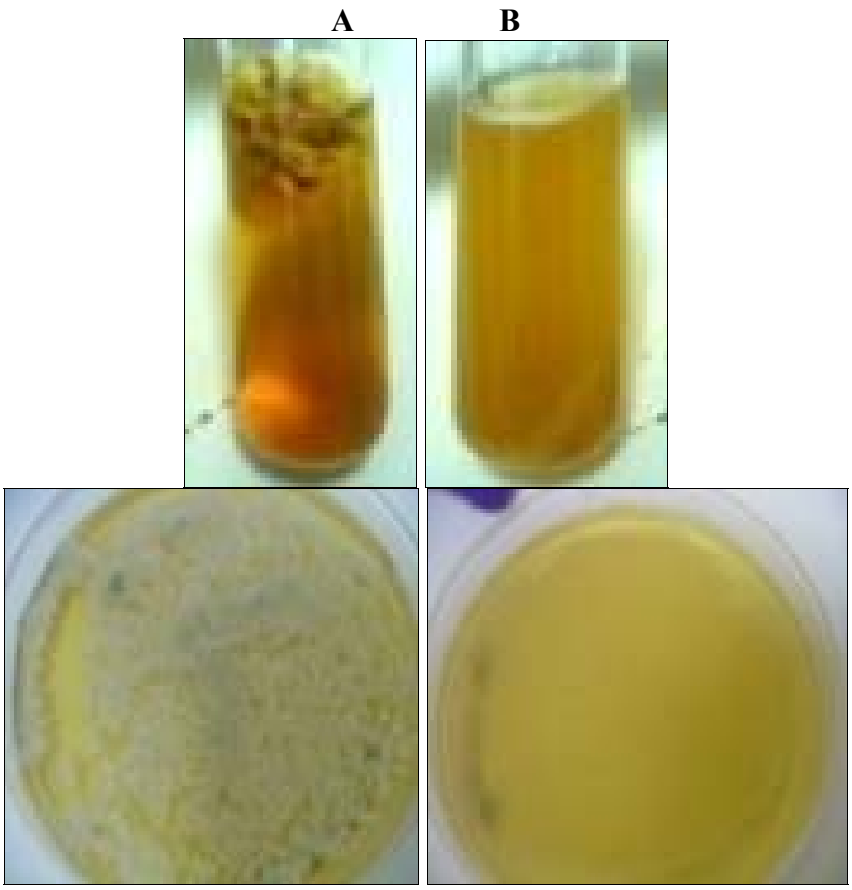


Figure 27. In vitro growth of *A. terreus* in PDB and PDA. Panels: A, control growth of cells; B, growth of cells treated with cellobio-oligosaccharides. All tubes and petri-dishes were photographed ten days after incubation at 30 °C.

Hydrolysis and Fermentation

Bagasse fermentation to ethanol

Sugarcane bagasse is a lignocellulosic material that on a mass basis contains 37%-43% cellulose, 20%-27% lignin and 18%-25% hemicellulose with the balance made-up by extractables and ash. Traditionally, ethanol comes from the fermentation of sugars (sugarcane juice) or starch (corn). The biomass leftover after the extraction of sugar from sugarcane is called bagasse, and this lignocellulosic material is generally burned in boilers at the sugar mills to generate steam.

This study resulted in the development of a technology to process cellulose from sugarcane bagasse into ethanol. The process involves the use of biomass, water and ammonium hydroxide in mass proportions of about 1: 8: 0.14 at 160°C for 60 min and requires at most seven steps for the conversion of biomass to ethanol: (1) pretreatment, (2) washing (optional), (3) grinding (optional), (4) pressing (5) separate hydrolysis and fermentation (SHF), (6) simultaneous saccharification and fermentation (SSF), and (7) distillation (Figure 28). Pretreatment breaks the biomass structure composed mostly of cellulose, hemicellulose, and lignin. The cellulose becomes available for saccharification as a result of the pretreatment. Washing removes inhibitors generated during the pretreatment step. Grinding increases the surface area of the biomass for saccharification. Pressing reduces the moisture content of biomass to allow higher solids loading. Saccharification is performed by a mixture of Novozyme 188 (Novozyme Inc.) and Spezyme CP (Genencor Inc.). Finally, glucose is consumed by yeast

(*Saccharomyces cerevisiae* D_5A ATCC 200062) under anaerobic conditions to generate alcohol. The initial concentration of solids was increased (10-30% dry solids) by feeding more biomass to the batch reactor.

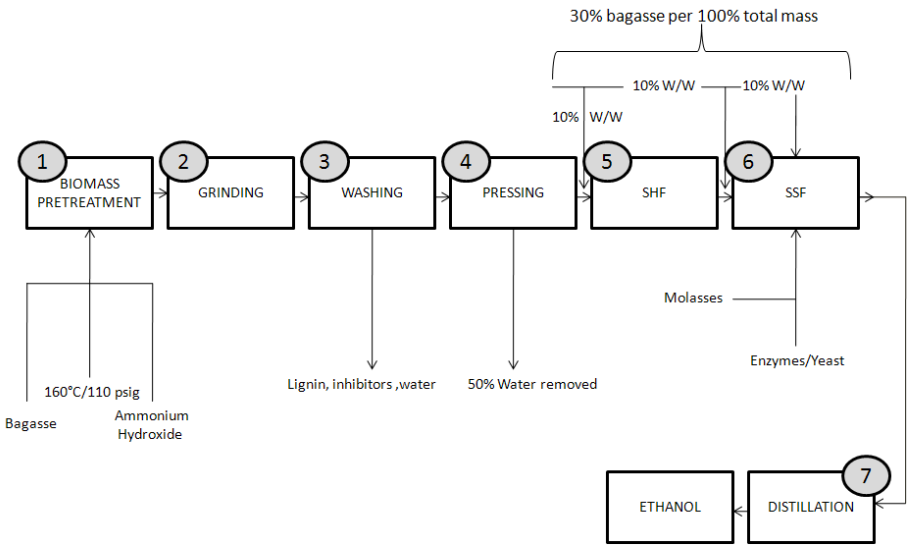


Figure 28. Flowchart for the Production of Ethanol from Sugarcane Bagasse.

Biomass composition was calculated before and after pretreatment following National Renewable Energy Laboratory's (NREL) Analytical Laboratory Procedure (LAP) for the determination of carbohydrates and lignin in biomass (Table 14).

Table 14. Composition analysis of untreated, ammonium hydroxide and water-treated sugarcane bagasse.

	Sugarcane Bagasse*		
Biomass Constituent	Untreated	Water†	Ammonium Hydroxide
Glucan (Cellulose)	38.4	60.5	63.6
Xylan (Hemicellulose)	24.1	13.3	24
Lignin	25	24.3	21.1

*= Compositions are given in mass percent units: grams of component per 100 g of dry biomass.
†= Sugarcane bagasse was treated with water only at 160°C for 1 hour as control.

SSF, Media Supplementation

Glucose levels obtained from either 10% or 30% dry solids represented at least 85% theoretical cellulose conversion. In order to enhance the fermentation efficiency a small amount of blackstrap molasses was added as a nutrient supplement. The composition of the molasses is given in Table 15. A 5% ethanol concentration was obtained at 30% solids with 5% molasses supplementation (Table 16). Percent ethanol yields were greater than 90% of theoretical obtainable from the glucan present in the fermentation.

Table 15. Composition of blackstrap molasses.

Sugar	Percent (g/100 g molasses)
Sucrose	25.3
Glucose	8.1
Fructose	9.5

Table 16 summarizes the concentrations of ethanol obtained after supplementation of fermentation broth with increasing amounts of blackstrap molasses at 30% solids loading of ammonia-treated bagasse. Experiments were performed at a laboratory scale (1L).

Approximately, a 32% increase in ethanol (from cellulose only) was observed when 5% (w/w) molasses was added to the fermentation broth regardless of enzyme loading. Less than 0.2% (w/w) of the glucose remained post SSF. Broth supplementation with 17% (w/w) molasses resulted in ethanol concentrations of 14% (w/w) and 10 % (w/w) for full strength and half strength enzyme loadings, respectively. Unfermented glucose was 4.6% (w/w) and 1.6% (w/w). The yeast strain used in this experiment has a 10% maximum tolerance to ethanol, hence the high concentration of unfermented sugars at 17% (w/w) broth supplementation with molasses.

Table 16: Ethanol production from dilute ammonia-treated bagasse and blackstrap molasses as supplemental feed.

Enzyme Loading (Spezyme CP and Novozyme 188)	Percent Molasses (w/w)					
	0		5		17	
	Percent (w/w)		Percent (w/w)		Percent (w/w)	
	Ethanol	Glucose*	Ethanol	Glucose*	Ethanol	Glucose*
Full Strength	3.9	0.1	5.7	0.2	13.5	4.6
Half Strength	3.4	0.1	5.1	0.2	10.4	1.6

*= Remaining glucose in solution post SSF

Ethanol titer increased with increasing molasses concentrations. Experiments were carried out at 30% solids (30 g dry bagasse/ 100 g total mass) with molasses being added to the fermentation mixture up to 15% (w/w). Two enzyme concentrations were also evaluated. Ethanol titers were higher with 15% (w/w) molasses supplementation and ranged from 10.4%-13.5%. Un-fermented sugars were observed at 10% (w/w) ethanol in the fermentation broth, an indication that the yeast (*Saccharomyces cerevisiae* ATCC 200062) had reached its alcohol tolerance level. Data is summarized in Table 17.

Table 17. Ethanol yields by media supplementation with blackstrap molasses.

	Percent (W/W)		
Pre-Treatment	Molasses	Glucose#	Ethanol
Ammonium Hydroxide†	0	0.1	3.8
Ammonium Hydroxide	5	0.2	5.6
Ammonium Hydroxide	15	4.6	13.5
Ammonium Hydroxide*	0	0.1	3.4
Ammonium Hydroxide	5	0.2	5.1
Ammonium Hydroxide	15	1.6	10.4

#= Percent glucose at the end of SSF
†= Enzymes added at a concentration of 60 FPU/g glucan (Spezyme CP) and 30 CBU/g glucan (Novozyme 188)
*= Enzymes added at a concentration of 30 FPU/g glucan (Spezyme CP) and 15 CBU/g glucan (Novozyme 188)

The dilute ammonia process was evaluated at a pilot level (Figure 29). In a normal run, 1.2 kg dry biomass, ammonium hydroxide and water were loaded into the reactor and heated to 160°C for 60 min. SSF studies were carried out in a bioreactor with a 30 L capacity. Both enzyme hydrolysis and ethanol yields were comparable to the ones obtained in the laboratory where SSF studies were carried out in 2L bioreactors. The composition analysis of ammonia-treated and water-treated bagasse before and after SSF with and without media supplementation with 5% (w/w) molasses at 10% and 30% (g dry biomass/100 g total weight) solids loading is summarized in Figures 30 and 31. Approximately, 4% (total mass) of the hemicellulose was lost to solution after pre-treatment with dilute ammonia and 10% with water pretreatment. More lignin (5%, total mass) was lost with dilute-ammonia than with water (1%, total mass) pre-treatment.

Most of the cellulose and hemicellulose were hydrolyzed by SSF, in particular, those in dilute ammonia-treated bagasse. Approximately, 10% (total mass) of cellulose remained as fiber compared to 33% (total mass) for water-treated bagasse at 10% solid loadings laboratory trials. Pilot tests, compared to laboratory tests, showed less cellulose being hydrolyzed at higher mass loadings, 16% (total mass) and 38% (total mass), respectively. At 30% solids loading, 27% (total mass) and 42% (total mass) of cellulose remained as fiber. This difference can be attributed to mixing conditions, spindle mixing versus tumbling at the pilot plant. Spezyme CP and Novozyme 188 contain both cellulases and hemicellulases thus the low percentages of hemicellulose seen on the fiber.

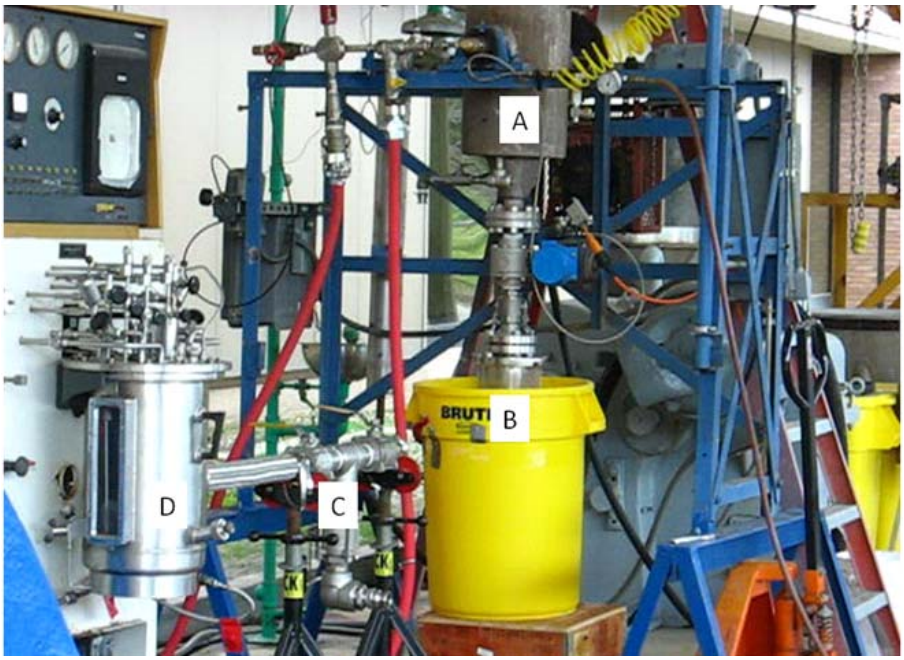


Figure 29. Pilot Plant Equipment Set-Up. (A) Reactor used during biomass pretreatment and separate hydrolysis and fermentation (SHF); (B) Double-walled stainless steel cylinder attached to the bottom of the reactor post pretreatment for the washing and removal of inhibitors (optional), (C) Flexibles stainless steel hose fitted to a T valve for the transfer of partially liquefied biomass to a 30 L bioreactor post SHF, (D) Bioreactor where liquefied biomass was collected in batches for the production of ethanol during simultaneous saccharification and fermentation (SSF).

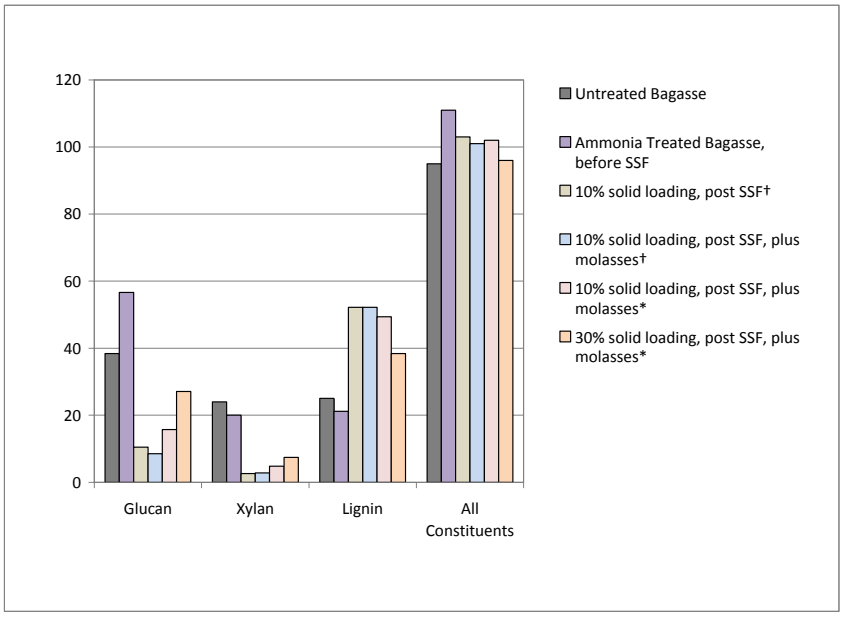


Figure 30: Composition Analysis of Ammonia-Treated Bagasse Post SSF.
Sugarcane bagasse was pre-treated with dilute ammonia. Ammonia-treated bagasse (10% and 30% solid loading) was hydrolyzed using Spezyme CP and Novozyme 188. Fermentation broth was supplemented with 5% (w/w) blackstrap molasses. *Saccharomyces cerevisiae* was used to convert cellulose to ethanol. Biomass constituents include ash, water and ethanol extractables, lignin, glucan, xylan, arabinan, and mannan. †= Laboratory Trials (1L); *= Pilot Trials (20L).

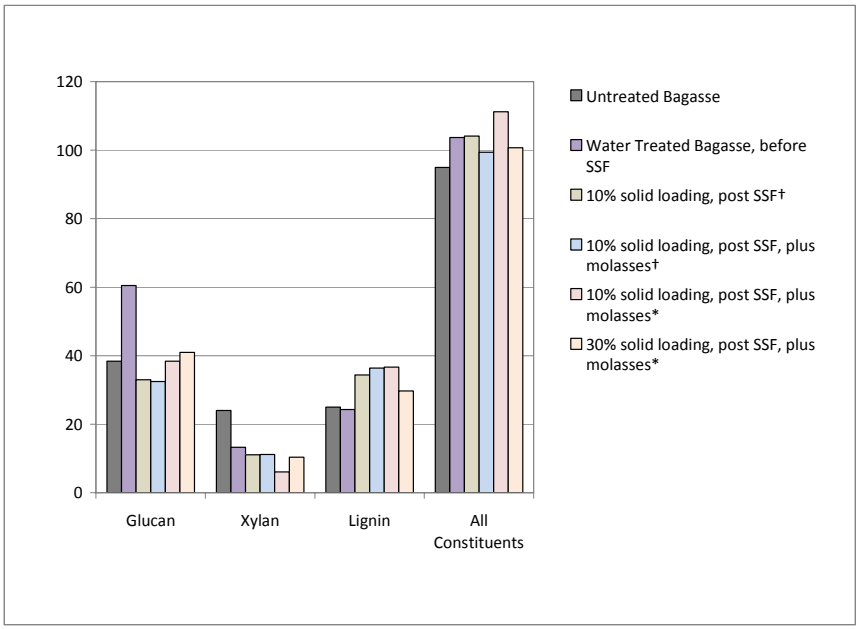


Figure 31: Composition Analysis of Water-Treated Bagasse Post SSF.
Sugarcane bagasse was pre-treated with water. Water-treated bagasse (10% and 30% solid loading) was hydrolyzed using Spezyme CP and Novozyme 188. Fermentation broth was supplemented with 5% (w/w) blackstrap molasses. *Saccharomyces cerevisiae* was used to convert cellulose to ethanol. Biomass constituents include ash, water and ethanol extractables, lignin, glucan, xylan, arabinan, and mannan. †= Laboratory Trials (1L); *= Pilot Trials (20L).

Good conversion of bagasse can be obtained using an SHF/SSF combination, where SHF is used to solubilize the pre-treated biomass and the hydrolysis is finished during the SSF, reducing end-product inhibition on the enzymes. Rapid fermentation was best achieved with solids loading of no more than 10 %w/w at a time, indicating a fed-batch fermentation is optimum. An enhancement of ethanol yields was achieved by supplementation with blackstrap molasses. The increase in ethanol was significantly beyond what would be expected just from the additional sugars that were added.

Improvement of SHF(xylan hydrolysis)

Sugar cane bagasse is an attractive waste substrate for ethanol bioconversion. Approximately 24 and 38 % (dry weight) of bagasse consists of hemicellulose and cellulose respectively and bioconversion of both fractions to ethanol must be considered for a viable process. We evaluated the degree of hydrolysis of pretreated bagasse with combinations of cellulase, β-glucosidase and hemicellulase. Ground bagasse was pretreated either by the AFEX process (2 NH_3: 1 biomass, 100 °C, 30 min; kindly provided by MBI) or with ammonium hydroxide (0.5 g NH_4OH of a 28 % (v/v) per g dry biomass; 160 °C, 60 min) and in both samples, the amounts of glucan and xylan fractions remained largely intact. The enzyme activities of 14 commercial enzyme preparations and supernatants of six laboratory grown fungi were determined. Four commercial preparations and four laboratory preparation with significant xylanase activity were evaluated for their ability to boost xylan hydrolysis when used in combination with cellulase and β-glucosidase (10 FPU: 20 CBU/g glucan). When evaluated at a 1 % glucan loading, one commercial enzyme preparation (added at 10 % level of total enzyme protein) boosted xylan hydrolysis by at least 40 % of both pretreated bagasse samples. The glucan hydrolysis was also boosted by the xylanase. Xylanase addition at 10 % protein level also improved hydrolysis of xylan and glucan fractions up to 10 % glucan loading (28 % solids loading). Significant xylanase activity in

enzyme cocktails appears to be required for greater hydrolysis of both glucan and xylan fractions of ammonia pretreated sugar cane bagasse.

The relatively high carbohydrate and low lignin content make bagasse an attractive substrate for ethanol production. Approximately 24 and 38 % (dry weight) of bagasse consists of hemicellulose and cellulose respectively and bioconversion of both fractions to ethanol must be considered for a viable process. Pretreatment of bagasse is necessary to reduce the recalcitrance to enzymatic hydrolysis. Previous studies have reported the pretreatment of sugar cane bagasse with either physical or chemical methods such as acid (Martin et al., 2002), steam (Kaar et al., 1998) or alkali (Fox et al., 1987; Holtzapple et al., 1991). Chemical pretreatments generally remove the hemicellulose or lignin fraction thereby promoting cellulose hydrolysis (Mosier et al., 2005). Dilute sulphuric acid has been widely used to pretreat sugar cane bagasse (Van Zyl et al., 1989; Martin et al., 2002) and results in the hemicellulose fraction being released as pentoses such as xylose and arabinose. Without detoxification, the subsequent fermentation can be seriously inhibited. Alkali pretreatment generally leaves the hemicellulose fraction relatively intact. The ammonia freeze explosion (AFEX) process is a particularly attractive pretreatment of sugar cane bagasse (Holtzapple et al., 1991) as ammonia can be potentially recycled while only some hemicellulose is removed and the formation of sugar degradation products are minimized (Mosier et al., 2005). Furthermore this process enables both the cellulose and hemicellulose fractions to be hydrolyzed enzymatically.

Table 18 shows the composition of sugar cane bagasse and the bagasse after treatment with the AFEX process or with ammonium hydroxide. The pretreatment process resulted in an increase in the glucan concentration especially in the ammonium hydroxide pretreated bagasse. Both pretreatment processes left the hemicellulose fraction of bagasse largely intact.

Table 18. Composition analysis (g/100 g dry biomass) of ammonium hydroxide- and AFEX- treated sugar cane bagasse

Component	**Raw sugar cane bagasse**	**Pretreated sugar cane bagasse**	
		0.02 g NH_3/ 100 g H_2O, 160 °C, 1 h	AFEX (2 NH_3: 1 biomass, 100 °C, 30 min)
Ash	4.2	2.8	10.0
Ethanol extractives	1.1	3.9	5.8
Acid soluble lignin	5.3	5.0	8.2
Acid insoluble lignin	19.7	16.1	14.4
Glucan	38.4	56.6	41.7
Xylan	24.1	24.0	20.4
Arabinan	1.9	1.2	1.2
Mannan	0	1.7	1.5

The cellulolytic activities of 14 commercial preparations are shown in the Appendix. Filter paper activity could only be detected in some samples and the highest activities were obtained in BioCat xylanase (sample 1), Spezyme CP (sample 8) and Fibrezyme LBL (sample 12). Samples showed a considerable range in CMCase (an indicator of endo-β-1,4-glucanase), avicelase (an indicator of exoglucanase) and β-glucanase activities. BioCat products showed high activities of the various cellulolytic enzymes (samples 1, 4). Novozym 188 (sample 5) as expected had the highest β-glucosidase activity.

Table 19 shows the hemicellulolytic specific activities of the 14 commercial samples. β-xylanase activities ranged between 10 and 2967 U/mg. Especially notable was the low β-xylanase activities of Novozym 188 (sample 5) and Spezyme CP (sample 8), two enzyme preparations commonly used in the hydrolysis of lignocellulose. The purpose of the analysis was to establish the hemicellulolytic enzyme profile that might be appropriate for addition to a cellulase/β-glucosidase enzyme mixture. The most suitable enzyme preparations were identified as BioCat xylanase, BioCat hemicellulase. *T. lanuginous* xylanase, Multifect xylanase, PowerPulp TX200A and FibreZyme LBL. These six preparations showed the highest xylanase specific activity. Furthermore BioCat xylanase, Multifect xylanase, and PowerPulp TX200A revealed specific β-xylosidase activity greater than one and BioCat xylanase and Multifect xylanase were found to have the highest α-arabinofuranosidase activity. These results suggest that BioCat xylanase, Multifect xylanase, PowerPulp TX200A and FibreZyme LBL might be the best hemicellulolytic enzyme preparations that should be added to a cellulase/β-glucosidase enzyme mixture in order to achieve the most effective hydrolysis of sugar cane bagasse to a mixture of hexoses and pentoses.

Table 19. Specific activities of Commercial Xylanases

Sample	**Enzyme preparation**	**β-xylanase** μmol/min/mg	**β-xylosidase** μmol/min/mg	**α-arabinofuranosidase** μmol/min/mg
1	BioCat xylanase 10 mg/ml	1235±57	5.87±0.31	5.07±0.45
2	BioCat hemicellulase 10 mg/ml	609±20	0.82±0.09	0.39±0.10
3	*T. lanuginosus* xylanase, 10 mg/ml	2967±18	0.11±0.09	1.18±0.07
4	BioCat cellulase, 10 mg/ml	36±1	0.80±0.10	0.55±0.06
5	Novozym 188	10±1	0.22±0	0.09±0.1
6	Multifect xylanase	209±10	4.90±0.58	3.21±0.40
7	*T. reesei* ATCC 26921 cellulase	20±1	2.11±0.23	1.29±0.05
8	Spezyme CP	15±2	0.56±0.02	0.38±0.04
9	Cellulase from *Aspergillus*	49±10	0.01±0.01	0.01±0
10	GC 220	20±2	0.06±0.01	0.02±0
11	PowerPulp TX200A	369±10	1.27±0.01	0.98±0.04
12	FibreZyme LBL	69±1	0.94±0.03	0.68±0.03
13	FibreZyme LWT	56±1	1.48±0.10	0.93±0.04
14	BioAce	22±1	1.33±0.03	0.88±0.01

The cellulolytic and hemicellulolytic enzyme profiles of number of selected fungi were also evaluated. The selection of the fungi was based on their properties determined in previous research. *A. carneus* ABO374 was isolated from soil in the Southern Cape region of South Africa and found to efficiently release reducing sugars from wheat straw. The supernatant of this strain cultivated on wheat straw also improved digestibility of wheat straw in in vitro and field sheep feeding trials (WH van Zyl, A. Botha,

CW Cruywagen & B. A. Prior. 2005. unpublished data). The *T. lanuginosus* strains SSBP and ATCC 34626 were shown to be very efficient producers of xylanases but the β-xylanase activity maintained up to 60 °C (Singh et al., 2000a; Singh et al., 2000b). *A. carneus* cultivated on AFEX pretreated sugar cane bagasse produced the highest specific activities of cellulolytic enzymes and β-glucosidase. *T. lanuginosus* strain SSBP produced lower cellulolytic specific activities when compared with the ATCC 34626 when cultivated under similar conditions. Most activities of cellulolytic enzymes were higher when strain ATCC 34626 was grown on beechwood xylan than AFEX-pretreated bagasse suggesting that the pure xylan is a better substrate for enzyme production. The highest specific β-xylanase activity was produced by strain ATCC 34626 when cultivated on beechwood xylan whereas lower activity was found when the *T. lanuginosus* strains were grown on AFEX-pretreated bagasse and very little β-xylanase was produced by *A. carneus*. These results also revealed that the *T. lanuginosus* strains SSBP and ATCC 34626 yielded the highest specific xylanase specific activities and these values are higher than those found in the most commercial samples with the exception of the commercial *T. lanuginosus* xylanase which is apparently a recombinant enzyme. *A. carneus* produced the highest activity of β-xylosidase and α-arabinofuranosidase whereas the *T. lanuginosus* strains produced much lower activities of these auxiliary enzymes as has been reported previously (Singh et al., 2000a).

In a simultaneous saccharification and fermentation (SSF) to ethanol process, the temperature is restricted to 30 °C if a yeast strain such as *S. cerevisiae* is used. Therefore the activities of the β-xylanase were evaluated at 50 °C (which is closer to optimum for many of the enzymes selected) and 30 °C (which would appropriate for a SSF process). The activity of most β-xylanases was lower at 30 °C than at 50 °C. However, the activities of Multifect, PowerPulp and FibreZymeLBL β-xylanases were only slightly lower or not at all at the lower temperature. The other activities of the commercial and laboratory produced β-xylanases were much lower at 30 °C than at 50 °C.

Spezyme CP is a commercial cellulolytic enzyme preparation produced by *Trichoderma reesei* and has been widely used in the hydrolysis of cellulose-rich biomass due to the dominance of endoglucanases and exoglucanases in the preparation. However this preparation lacks adequate β-glucosidase activity (Lynd et al., 2002) to achieve complete hydrolysis to glucose and as a result there is an accumulation of cellobiose. Therefore Spezyme CP is usually supplemented with β-glucosidase in order to promote complete hydrolysis. In the literature the ratios of cellulase to β-glucosidase have ranged widely and have depended upon the nature of the lignocellulose material to be hydrolysed. For example Berlin et al. (2005) used a ratio of 1 FPU: 2CBU to hydrolyse various softwood substrates, Martin et al. (2002) used a ratio of 1 FPU: 5.4 CBU while the NREL procedure (LAP-009) recommended a ratio of approximately 1 FPU: 1 pNPGU to hydrolyze stream pretreated sugar cane bagasse. A ratio of 1 FPU: 2 CBU was selected for hydrolysis experiments here and the ratio was based on the reported commercial enzyme activities of 60 FPU/ml for Spezyme CP and 282 CBU/ml for Novozym 188. Based on our data a ratio of 1 FPU Spezyme CP: 3.3 pNPGU Novozym 188 is recommended.

Table 20. Sugar release from ammonium hydroxide- and AFEX pretreated sugar cane bagasse (at 1 % glucan level) by Spezyme CP and Novozym 188 (1:2 ratio activity)

Enzyme activity Spezyme/Novozyme FPU:CBU/g glucan	Sugar concentration (g/l)					% hydrolysis	
	cellobiose	glucose	xylose	arabinose	total*	glucan	xylan
Ammonium-hydroxide pretreated							
10:20	0	3.11	2.09	0	5.2±0.27	28	41
30:60	0	5.73	3.16	0	8.89±0.76	52	62
60:120	0	7.64	3.8	0	11.44±0.43	68	75
AFEX-pretreated							
10:20	0	3.68	2.87	0.98	7.53±0.54	33	65
30:60	0	6.14	3.78	1.19	11.11±1.13	55	84
60:120	0	7.51	4.36	1.17	13.04±0.37	68	94
Avicel							
10:20	0	7.31	0	0	7.31±0.65	66	
30:60	0	8.27	0	0	8.27±0.40	74	
60:120	0	8.68	0	0	8.68±0.20	78	

*mean of triplicate determinations ± standard deviation

Table 20 shows the effect of three levels of Spezyme CP/Novozym 188 (in an activity ratio of 1:2) on the hydrolysis of ammonium hydroxide-pretreated and AFEX-pretreated bagasse and Avicel. As the enzyme level increased, greater amounts of glucose and xylose were released from the bagasse samples and glucose from the Avicel. No cellobiose was detected in any of the samples suggesting that the β-glucosidase activity was not limiting in any of the enzyme mixtures. Arabinose was only released from the AFEX-pretreated bagasse. At a 10 FPU: 20 CBU/ g glucan ratio, the greatest amount of glucose was released from Avicel followed by the AFEX-pretreated bagasse. At the lowest enzyme level only 28 % and 33 % of the glucan in respective NH_4OH- and AFEX-pretreated bagasse samples were hydrolyzed to glucose whereas 66 % of the Avicel glucan was hydrolyzed suggesting that some glucan was inaccessible in the bagasse samples to enzyme hydrolysis. At the highest enzyme level (60 FPU: 120 CBU), 68 % and 78 % of the glucan was hydrolyzed in the respective bagasse samples and Avicel samples. Increasing the level of enzyme appeared to have a much lower impact on the hydrolysis of the Avicel sample that the bagasse samples. This might be due to a synergistic effect of the other enzymes present in the Spezyme CP/Novozym 188 acting on the cellulose and hemicellulose components in bagasse.

Table 21. Effect of addition of commercial xylanase preparation to hydrolysis of ammonium hydroxide pretreated sugar cane bagasse by Spezyme CP (10FPU/g glucan)/Novozym 188 (20CBU/g glucan)

Enzyme preparation	% protein loading	Sugar (g/l)					% hydrolysis	
		Cellobiose	Glucose	Xylose	Arabinose	Total*	Glucan	Xylan
None	0	ND	3.49	1.94	0.26	5.64±0.34	31	43
Multifect	10	ND	4.3	2.72	ND	7.02±0.84	39	54
	50	ND	4.76	2.9	ND	7.66±1.43	43	57
Power pulp	10	ND	2.57	1.67	0.18	4.42±0.45	23	37
	50	ND	3.88	2.19	0.18	6.24±0.64	35	47
FibreZyme LBL	10	ND	3.31	1.97	0.17	5.44±0.27	30	42
	50	ND	4.88	2.55	0.55	7.98±1.20	44	61
BioCat xylanase	10	ND	3.7	2.22	0.13	6.05±0.45	33	46
	50	ND	6.1	2.83	0.28	9.21±0.19	55	61

ND: not detected
*mean of triplicate determinations ± standard deviation

Table 22. Effect of addition of commercial xylanase preparation to hydrolysis of AFEX- pretreated sugar cane bagasse by Spezyme CP (10FPU/g glucan)/Novozyme 188 (20CBU/g glucan) (triplicate determinations)

Enzyme preparation	% protein loading	Sugar (g/l)					% hydrolysis	
		Cellobiose	Glucose	Xylose	Arabinose	Total	Glucan	Xylan
None	0	ND	4.32	2.86	0.38	7.57±0.30	39	55
Multifect	10	ND	4.94	3.99	0.66	9.59±0.51	44	62
	50	ND	6.77	4.65	0.73	12.15±1.28	61	91
Power pulp	10	ND	4.79	3.29	0.29	8.37±0.82	43	61
	50	0.05	5.98	3.78	0.24	10.05±2.31	54	68
FibreZyme LBL	10	0.01	4.49	3.28	0.24	8.02±0.10	41	60
	50	0.01	6.16	3.79	0.46	10.43±1.20	55	72
BioCat xylanase	10	ND	5.91	3.96	0.29	10.16±0.58	53	72
	50	0.09	7.97	4.3	0.34	12.69±1.56	73	79

ND: not detected
*mean of triplicate determinations ± standard deviation

Four xylanase preparations with suitable activity profiles were evaluated for their ability to boost the hydrolysis by Spezyme CP and Novozym 188 (10 FPU: 20 CBU/g glucan) of NH_4OH- (Table 21) and AFEX-pretreated bagasse and Avicel (data not shown). These enzyme preparations contained multiple cellulolytic and hemicellulolytic activities. Therefore the protein concentration was used as the basis to supplement the Spezyme CP/Novozym mixture instead of using the activity of a single enzyme such as xylanase. At a 10 % protein level, Multifect and BioCat xylanase had a significant increase in total sugar release compared to the control whereas with PowerPulp xylanase and FibreZyme LBL xylanase no increase was found. When the enzyme preparations were added at 50 % protein level, significant increases were observed in all instances. No cellobiose was detected. In spite of the hydrolysis increasing from 31 % to respectively 43, 44 and 55 % when Multifect, FibreZyme LBL and BioCat xylanases were added at the 50 % protein level, the degree of hydrolysis remained low. The hydrolysis of AFEX-pretreated bagasse was boosted significantly by the addition of a 10 % level of Multifect and BioCat xylanase to Spezyme CP and Novozym 188 (10 FPU: 20 CBU/glucan) and at a 50 % level, all

the xylanase preparation increased significantly (Table 22). Interestingly the release of glucose also increased markedly and the degree of the glucan hydrolysis increased from 39 % to respectively 61 % and 73 % by the addition of Multifect and BioCat xylanase at the 50 % protein level.

An analysis of the relationship between xylanase activity added and sugar release found that increasing units of enzyme activity had the greatest impact on the release of glucose and less on xylose (to be evaluated statistically).

The interaction between xylanase and the release of glucose from glucan was evaluated further by adding Multifect and PowerPulp xylanases to Spezyme CP and Novozym 188 in the hydrolysis of Avicel. Both Multifect and PowerPulp xylanases boosted the hydrolysis significantly when added at a 50 % protein level whereas at 10 % protein level only the Power Pulp xylanase had a significant impact (Table 23). PowerPulp and Multifect xylanase added at the 50 % protein level increased the hydrolysis from 65 % to 80 % and 76 % respectively. Both xylanase preparations had relatively low cellulolytic enzyme activities and therefore these results suggest that the xylanase might act synergistically in the hydrolysis of cellulose by cellulase. This point needs to be confirmed by further investigation.

Table 23. Effect of substrate loading on hydrolysis of AFEX and NH_3-pretreated sugar cane bagasse by Spezyme (10 FPU/g glucan) and Novozym 188 (20 CBU/g glucan) with or without Multifect xylanase added at 10 % protein (mean of triplicate determination)

% Glucan Loading	Sugar (g/l)					% hydrolysis	
	Cellobiose	Glucose	Xylose	Arabinose	Total*	glucan	xylan
Ammonium hydroxide pretreatment with xylanase							
1	0.05	4.27	2.77	0.19	7.29±0.58	39	58
2	ND	9.21	6.05	0.49	15.75±0.75	41	65
4	0.07	22.93	13.21	0.99	37.21±1.30	52	70
AFEX-pretreatment							
1	0.06	5.73	4.51	0.42	10.71±1.21	53	84
2	0.32	14.23	8.74	1.05	24.34±0.55	67	83
5	0.91	28.47	19.45	2.57	51.41±2.83	55	75
10	5.43	46.39	34.56	4.5	90.89±0.75	52	66
Without xylanase							
	0.06	3.76	2.32	0.18	6.32±0.37	35	49
	ND	6.61	4.26	0.37	11.24±1.40	30	46
	ND	17.79	9.93	0.85	28.57±2.34	40	53
	0.22	5.64	3.74	0.32	9.92±0.66	55	69
	0.19	11.34	7.16	0.71	19.40±0.68	53	67
	1.89	26.14	16.48	1.88	46.39±0.64	54	62
	9.78	38.3	27.53	3.26	78.87±2.28	52	52

ND: not detected
*mean of triplicate determinations ± standard deviation

The impact of laboratory-produced enzymes added at a 10 % protein level on the hydrolysis by Spezyme CP and Novozyme 188 is shown in Table 24. Enzymes produced by *A. carneus* failed to boost the hydrolysis of NH_4OH- and AFEX-pretreated bagasse. The enzyme preparation produced by

T. lanuginosus ATCC 34626 grown on beechwood xylan was the most effective of the various laboratory produced enzyme preparations in increasing the amount of glucose, xylose and total sugar released. The percent hydrolysis of the glucan fraction of NH_4OH- and AFEX-pretreated bagasse increased respectively from 32 % to 40 % and from 49 % to 54 %. As shown in the studies with the commercial xylanase preparations, greater amounts of sugar was released from the AFEX-pretreated bagasse than the NH_4OH-pretreated bagasse when the laboratory-produced enzymes were added to Spezyme CP and Novozym 188 suggesting that the AFEX-pretreated bagasse is more easily hydrolyzed.

Table 24. Effect of addition of concentrated lab xylanase preparations (10 % protein) to hydrolysis of pretreated sugar cane bagasse (1 % glucan) by Spezyme CP (10FPU/g glucan)/Novozyme 188 (20CBU/g glucan)

Organism	Growth conditions	Sugar (g/l)					% hydrolysis	
		Cellobiose	Glucose	Xylose	Arabinose	Total*	Glucan	Xylan
Ammonium hydroxide pretreated bagasse								
A. carneus ABO372	AFEX	0.06	3.23	2.32	0.18	5.80±0.43	30	49
T. lanuginosus SSBP	AFEX	0.07	4.17	3.16	0.22	7.62±0.14	39	69
T. lanuginosus ATCC 34626	AFEX	ND	3.74	2.82	0.3	6.87±0.78	34	62
T. lanuginosus ATCC 34626	Beechwood	ND	4.41	3.41	0.25	8.07±0.91	40	72
None		0.03	3.53	2.2	0.17	5.93±0.30	32	47
AFEX pretreated bagasse								
A. carneus ABO372	AFEX	0.14	5	4.02	0.3	9.46±0.48	48	73
T. lanuginosus SSBP	AFEX	0.12	5.51	4.1	0.27	9.99±0.96	52	74
T. lanuginosus ATCC 34626	AFEX	0.01	5.87	4	0.28	10.16±0.93	53	73
T. lanuginosus ATCC 34626	Beechwood	ND	6.01	4.55	0.25	10.82±0.71	54	82
None		ND	5.48	3.94	0.27	9.69±0.66	49	72
Avicel								
A. carneus ABO372	AFEX	0.08	9.53	ND	ND	9.61±0.44	88	
T. lanuginosus SSBP	AFEX	0.11	10.75	ND	ND	10.86±0.97	100	
T. lanuginosus ATCC 34626	AFEX	0.03	7.68	ND	ND	7.71±0.41	70	
T. lanuginosus ATCC 34626	Beechwood	0.08	8.59	ND	ND	8.67±0.18	79	
None		0.08	8.83	ND	ND	8.91±0.33	82	

Growth conditions: Fungus grown either on AFEX pretreated bagasse or beechwood xylan
ND: Not detected
*mean of triplicate determinations ± standard deviation

As the Multifect xylanase was shown to be one of the most effective xylanase preparations in boosting the hydrolysis of bagasse, the effect on substrate loading on the hydrolysis of NH_4OH- and AFEX-pretreated bagasse by Spezyme CP and Novozym 188 with and without Multifect xylanase was

evaluated (Table 25). At all substrate loadings, there were greater amounts of xylose, arabinose and glucose released when xylanase was present. Due to the nature of the NH_4OH-pretreated bagasse, a maximum substrate loading of only 4 % could be tested whereas the AFEX-pretreated bagasse could be tested up to 10 % substrate loading. However, it was apparent that greater amounts of both glucose and xylose were released from the AFEX-pretreated bagasse than the NH_4OH-pretreated bagasse. Surprisingly the % hydrolysis of the glucan fraction of bagasse appeared to be unaffected by the load increase but the xylan hydrolysis declined with an increase in loading in the AFEX-pretreated bagasse. Notable was the greater accumulation of cellobiose with an increase in the loading of the AFEX-pretreated bagasse. Furthermore the accumulation was greater in the absence of Multifect xylanase. This suggests that additional β-glucosidase activity might be necessary at higher loadings.

Table 25. Effect of substrate loading on hydrolysis of AFEX and NH3-pretreated sugar cane bagasse by Spezyme (10 FPU/g glucan) and Novozyme 188 (20 CBU/g glucan) with or without Multifect xylanase added at 10 % protein (mean of triplicate determination)

% glucan loading	**Sugar (g/l)**					**% hydrolysis**	
	Cellobiose	Glucose	Xylose	Arabinose	Total*	glucan	xylan
Ammonium hydroxide pretreatment with xylanase							
1	0.05	4.27	2.77	0.19	7.29±0.58	39	58
2	ND	9.21	6.05	0.49	15.75±0.75	41	65
4	0.07	22.93	13.21	0.99	37.21±1.30	52	70
AFEX-pretreatment							
1	0.06	5.73	4.51	0.42	10.71±1.21	53	84
2	0.32	14.23	8.74	1.05	24.34±0.55	67	83
5	0.91	28.47	19.45	2.57	51.41±2.83	55	75
10	5.43	46.39	34.56	4.5	90.89±0.75	52	66
Without xylanase							
	0.06	3.76	2.32	0.18	6.32±0.37	35	49
	ND	6.61	4.26	0.37	11.24±1.40	30	46
	ND	17.79	9.93	0.85	28.57±2.34	40	53
	0.22	5.64	3.74	0.32	9.92±0.66	55	69
	0.19	11.34	7.16	0.71	19.40±0.68	53	67
	1.89	26.14	16.48	1.88	46.39±0.64	54	62
	9.78	38.3	27.53	3.26	78.87±2.28	52	52

ND: not detected

*mean of triplicate determinations ± standard deviation

In conclusion, enzyme preparations have been assayed for their activities and certain preparations have been identified as being most suitable for use in hydrolysis experiments. Future work is required to evaluate if the addition of xylanases to cellulase/β-glucosidase enzyme mixtures can improve the rate of hydrolysis of AFEX-treated and ammonia-treated sugar cane bagasse as well as if the levels of cellulase/β-glucosidase enzyme mixture can be reduced in efficient bagasse hydrolysis.

Succinic acid production from biomass derived sugars

Succinic acid had been identified as a potential by-product for a biorefinery if sugar containing feedstocks from lignocellulose could be produced at a low enough price.

We tested whether concentrated, biomass derived sugars could be used in a fed-batch mode in an attempt to lower the overall medium additions. Cane leaf matter (CLM) for the preparation of sugar hydrolysates. Conditions for the pretreatment of CLM using the AFEX process and enzyme hydrolysis were established. Two biomass loading concentrations were tested during hydrolyses and the hydrolysates were used "as is", i.e. without concentration of the syrup, in subsequent succinic fermentations. Higher biomass loadings reduced glucose and xylose yields by 11%, but the higher sugar concentrations were favorable for succinic acid fermentation. All fermentations consumed all sugars, required no additional growth factor supplement, and reached titers of 37g/l for the lower biomass loading and 46g/l succinic acid for the higher biomass loading. The succinic yields were 110 and 101 g succinic / 100 g sugar, respectively (theoretical maximum 112).

It was known that the primary limiting factor in succinic acid fermentations is a lack of reducing equivalents. We had demonstrated by metabolic flux analysis (MFA) that expression of a heterologous gene from the pentose phosphate pathway altered carbon flow in *A. succinogenes* and enhanced yields by 4%. We tested the effect of expression of the same gene under standard fermentation conditions. Surprisingly, under these conditions, the transformed strain showed the same yields as the control, but with an increase in titer of 7%. The titer of 67.4g/L is the highest we have seen in this size vessel with this medium.

Fermentation with an FZ45 transformant, carrying a plasmid expressing two genes, one that was expected to augment reducing power and one that channels more carbon towards the reverse TCA cycle, was performed. MFA analysis showed no further enhancement over expression of the first gene only. FZ45 fermentations are characterized by a short growth phase. Peak cell-density was lost soon after growth is completed, as judged by OD measurements. It was assumed that succinic acid production could be enhanced if peak cell-mass could be maintained longer. Literature searches identified some candidate metabolites that may be responsible for cell loss. Two genes encoding enzymatic activities that catabolize the potentially harmful metabolites to products of the central metabolism were cloned. FZ45 transformants expressing one and both of these two genes were generated.

Fermentations using Molasses

Small scale growth studies with FZ45 showed that molasses can partially substitute for other nutrients. Glucose fed-batch fermentations were performed with reduced nutrient – molasses medium. All sucrose and fructose from the molasses was consumed. Succinic titers reached 61g/l in fermentations without yeast extract, and 83g/l for a fermentation with intermediate yeast extract as medium addition. The yields varied between 87% and 97% [g succinic per g total substrate] in the no-yeast-extract fermentations, for unknown reasons. 97% is close to standard fermentation yields. The fermentation with intermediate yeast extract additions showed 104% yield, which represents a high yield under these conditions.

In conclusion, *A. succinogenes* is able to ferment the sugar sources in molasses and that molasses provides some beneficial nutrient, which allows lowering of other complex nutrients. The speed and amount of biomass produced in the fermentations with intermediate yeast extract levels, which reached more than twice the density seen in medium without molasses (OD660 of 12 vs. ~5), attest to the

beneficial effect of molasses. Additional batch fermentations will be performed to assess the benefits of molasses, which will include MFA analyses.

Fermentations using stillage

Yeast fermentations produce glycerol as an often undesirable by-product, which is carried over into stillage. Use of stillage as a nutrient source for succinic fermentations was successfully demonstrated under previous efforts. We had also shown that the glycerol from yeast fermentation is consumed and converted to succinic acid in the early stages of glucose fermentations, but not in later stages and not at all in fermentations with C5 sugars. We identified and cloned three genes encoding enzymatic activities involved in glycerol metabolism. FZ45 transformants were generated and these strains were used in glycerol fed-batch fermentations. One of the genes had no effect on glycerol consumptions. The results for two genes and the control are shown in Table 26.

Table 26: Glycerol Fed-Batch with recombinant FZ45 strains.

Strain	Glycerol consumed [g]	Succinic Acid Titer [g/L]
FZ45/vector (control)	45.6	71.4
FZ45/pJR795.22	49.0	71.4
FZ45/pJR762.99	60.1	73.4

Both transformants consumed more glycerol than the vector control. Fermentation profiles showed that glycerol is consumed for extended times in the transformed strains. Glycerol consumption increased in strain FZ45/pJR762.99 by 31.7%, which also raised succinic titers slightly.

Strain Development

Succinate Utilization

Metabolic engineering of Z. rouxii

Expression of succinic acid pathway genes in an acid tolerant yeast was not sufficient to achieve re-routing of carbon away from ethanol and towards TCA cycle intermediates. We attempted to decrease ethanol production in this yeast strain through screening with a suicide substrate to generate alcohol dehydrogenase (*adh*-) deficient mutants. Strains were identified that showed more than 5-fold reduction in alcohol dehydrogenase assays of cell lysates. However, upon culturing, these strains continued to produce ethanol at the same level as the parent strain.

Two yeast mutant strains were obtained from ATCC that carry mutations preventing ethanol production. Only one of these strains exhibited this phenotype upon culturing in our laboratory. This strain cannot ferment glucose, and a screen for glucose fermenting derivatives was performed. 57 glucose fermenting strains were identified; two of them did not produce ethanol upon culturing. We plan to transform these strains with succinic acid pathway genes and genes that channel carbon from pyruvate towards the TCA cycle. Upon culturing, metabolites such as pyruvate, lactate, acetate, ethanol, succinic acid, malate and fumarate will be measured by HPLC and compared with the parental control.

Isolation of New Organisms

We obtained eight acid tolerant yeasts from NREL. The genotypes of these strains are not known. These strains were cultured, archived, and their metabolite profiles were established. One of them showed reduced ethanol production and produced some succinic acid. The strain appears to be a haploid. We did not pursue work on this strain, as carbon re-routing with characterized strains was unsuccessful successful.

We began culturing and assessing viability of rumen isolates from our collection. We are interested in strains with improved growth and productivity as compared to the currently favored strain. Maintaining productivity at a slightly lower pH would be favored. Literature searches were performed to find genes encoding enzymes that catalyze the conversion of the carboxylic acid groups in succinic acid to the aldehyde or alcohol.

Genes encoding *succinate semialdehyde-dehydrogenases* are known from several organisms, but all catalyze the direction from the aldehyde to the acid, and not the reverse direction, which we desire. This task was revised to looking for a fumarate or malate modifying activity and searching for an enzyme that recognizes a different substrate but may show low affinity for succinate as well.

An enzyme from *Nocardia,* carboxylic acid reductase (Car), was characterized in Dr. Rosazza's laboratory at the University of Iowa, and shown to convert aromatic carboxylic acids to the corresponding aldehyde. An *E. coli* vector expressing this enzyme was sent. The Car activity was induced and enzymatic activity was determined in cell lysates as described (He *et al*., 2004, Applied and Environmental Microbiology **70**, 1874-1881).

We observed the same specific activity for the natural substrate, benzoate, as published. Specific activities towards other substrates are listed in Table 27.

Table 27: Relative activity of recombinant Car towards non-aromatic Substrates.

Substrate	Relative Activity
Benzoate	100%
Fumarate	0%
Malate	50%
Succinate	30%

These assays showed that succinate was recognized as substrate. The gene encoding Car was cloned into our shuttle vector under a strong *A. succinogenes* promoter. Cell lysates, prepared from transformed FZ45 cells, showed no Car activity for any of the substrates described. We verified presence of the correct plasmid in the cells, repeated the transformation, but obtained the same, negative result. It is known that Car requires a posttranslational modification for activity, and *E. coli* cells appear to be capable of performing this modification (Rosazza, personal communication). We assume that *A. succinogenes* does not have the necessary modifying enzyme, and therefore only inactive Car protein was produced.

Glucose/xylose utilizing yeast

All biomass contains 3 main components: lignin, cellulose and hemicellulose. The lignin can be easily separated, but the similarity between the carbohydrate polymers, cellulose [glucose] and hemicellulose [xylose] makes it economically unfeasible to separate them for ethanolic fermentation. There is a

major obstacle to co-fermenting the xylose and glucose to ethanol. The presence of xylose (and xylose derived products) inhibits the yeast from converting glucose to ethanol; while the presence of glucose in the medium inhibits, via a separate process, the fermentation of xylose to ethanol. Genetically, this appears to be a control problem. If one can modify the "operon" that controls ethanol production from xylose or glucose, then one can develop a simultaneous process for the conversion of both sugars to ethanol. However, the genetics of most industrially important microbial species have not been defined. Therefore, site-directed mutagenesis is not a viable option and chemical mutagens due to their non-selectivity tend to produce multiple mutations.

VUV mutagenesis at the Pohang Light Source has been used to produce dextran-sucrase strains of Leuconostoc with 30 times more activity than the parent strain[1]. This was through use of an indirect process using backscatter radiation from an aluminum plate set at an angle to the synchrotron beam. Louisiana State University's Center for Microstructures and Devices (CAMD), with its X-ray lithography scanners offered a more direct approach allowing control of both exposure time and beam energy to expose samples for direct mutagenesis.

The synchrotron ring at LSU-CAMD was used to develop protocols for mutating the yeast *Pichia stipitis. Pichia stipitis* is a yeast that can convert either glucose or xylose to ethanol. The goal is to alter this organism such that it can utilize xylose to produce ethanol in the presence of glucose. An altered Pichia would be of value to a current research program at the Audubon Sugar Institute for conversion of lignocellulose to ethanol. In order to validate mutation protocols using synchrotron light, damage produced by controlled X-ray exposure was first visualized on extracted, dried supercoiled plasmid DNA and then mutants constructed using irradiated DNA on a known from a control organism, *Leuconostoc mesenteroides*. Finally, dried Pichia was mutated and screened for the desired mutants.

The use of vacuum-uv to produce mutations is not new. Early work by Ito et al,1984 on mammalian cells and the direct radiation of hydrated DNA by Folkard et al, 2002 and the production of industrial mutants using vuv by Kim et al, 1997 have shown the potential utility of this procedure in biological research. In theory, the use of x-rays will produce a large number of non-revertable (single and double stranded break) mutations. Unlike ultraviolet mutation, there will not be any dimerization mutants produced by this technique. The challenge faced was to produce mutations without concurrent oxidation damage or DNA repair by cell systems in order to maximize mutants from a biological system with a poorly understood genetic system.

The effect of absorbers on the radiation power spectrum for typical exposure conditions at CAMD (electron energy E = 1.3 GeV, distance scanner to source point R = 10m, radius of curvature for the bending magnet r_0 = 2.928 m, electron current I = 100 mA, horizontal acceptance = 10 mrad) is shown in Fig. 32.

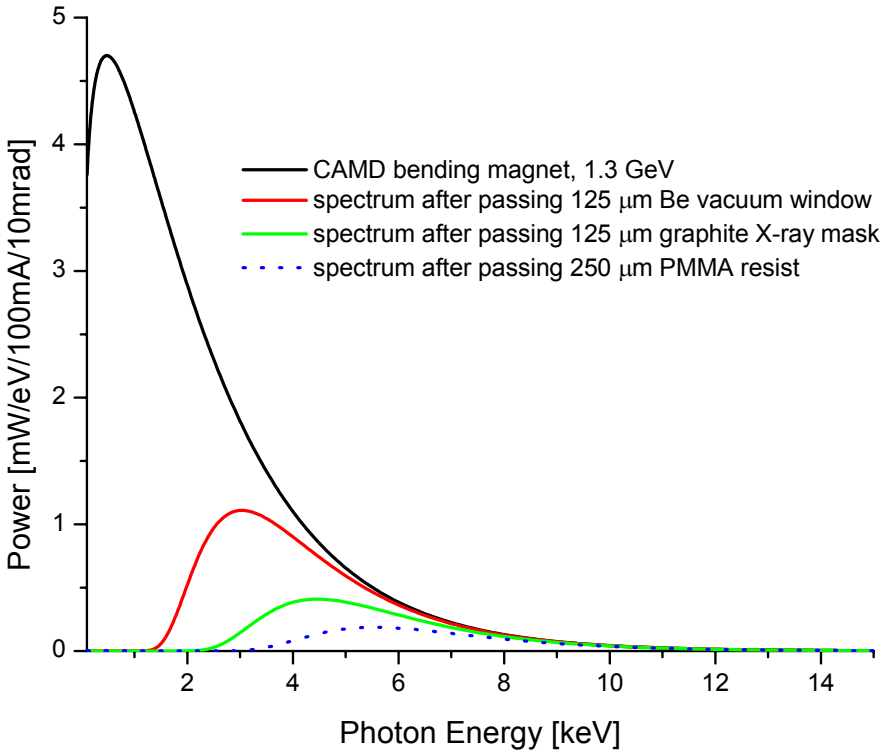


Figure. 32: Power spectrum after different absorber being inserted in the optical path in a typical DXRL exposure at CAMD. Low Z absorber materials cut-off the transmitted power spectrum at low photon energies resulting in an effectively harder X-ray exposure spectrum.

The low energy portion of the white light spectrum (below ~ 1.5 keV photon energy) is effectively absorbed by the 125 μm thick Be vacuum window. The Be window shifts the maximum of the spectrum to higher photon energies and reduces the intensity significantly. To increase the hardness of the SR spectrum and to determine a kill curve, varying thicknesses of aluminum foil were used, in parallel, to generate the best possible chance for mutants. An initial exposure series was conducted to test for the ability to recover the organism from the test cells, using a range of aluminum absorbers to harden and narrow the X-ray spectrum. Exposures were conducted under a helium atmosphere to minimize oxidative damage. For the first sequence a timed exposure of 20 sec was used (Table 28). One run with a different cycle length was tested (Table 29). Better results were obtained when a longer cycle time was used. Scanner travel above and below the sample holder was from -50 mm to +50 mm and the number of full cycles were counted. Both exposure time and absorber thickness versus cell recovery were determined in order to establish the range of exposure required for maximum mutagenesis.

Table 28: Effect of absorber thickness on cell kill by synchrotron radiation.

Time of Exposure (s)	Cells recovered	Absorber thickness (µm)	Beam Current (ma)
0	450,000	n/a	n/a
20	176,000	1,120	110
20	460,000	560	173
20	310,000	280	171
20	470,000	140	165
20	550,00	70	162
20	1	35	159

Table 29: Effect of cycle time on cell kill by synchrotron radiation.

Time of Exposure (s)	Cells recovered	Absorber thickness (µm)	Beam Current (ma)
20	460,000	560	173
80	43,000	560	108

The desired range is that amount of exposure which will produce a 99% kill on the exposed cells. After establishing proper absorber thickness and exposure range, DNA containing a supercoiled [non-replicating] DNA from a known plasmid was isolated and subjected to the same level of synchrotron radiation. Such DNA is extremely dry [free of associated water] and with the use of SR for such samples the secondary photolytic effects of X-rays on water with the consequent release of damaging radicals such as OH are eliminated[·] . Agarose gel profiles of this DNA showed decreasing amounts of supercoiled DNA with increasing exposure time. This is indicative of radiation induced breakage causing unwinding of the plasmid (Figure 33). SR was then used to produce mutations in DNA obtained from *Leuconostoc mesenteroides,* which following exposure to synchrotron radiation was transformed into *E. coli* cells and monitored for dextran production. Dextransucrase containing cells, determined by colony size and appearance (Figure 34) were enumerated. There was a significant increase in the number of dextransucrase expressing cells after radiation exposure (Table 30).

Table 30. Colony counts for dextransucrase expression on LBSucrose agar plates

Sample Name	Colony size		
	Big (.2-1.0 mm, slimy)	Middle	Small
N1	419	339	6
N2	221	217	47
DSRN	72	71	31
DSRN1	10	16	40
DSRN3	40	48	30

Dextransucrase expression test on LB Sucrose (3%, w/v) + Kanamycin (50 µg/mL) agar media. IPTG + X-gal (70µL) had been applied on the surface of plate before 50 µL of product from transformed cells plated. It is hard to distinguish the limit between middle and small size of colonies.

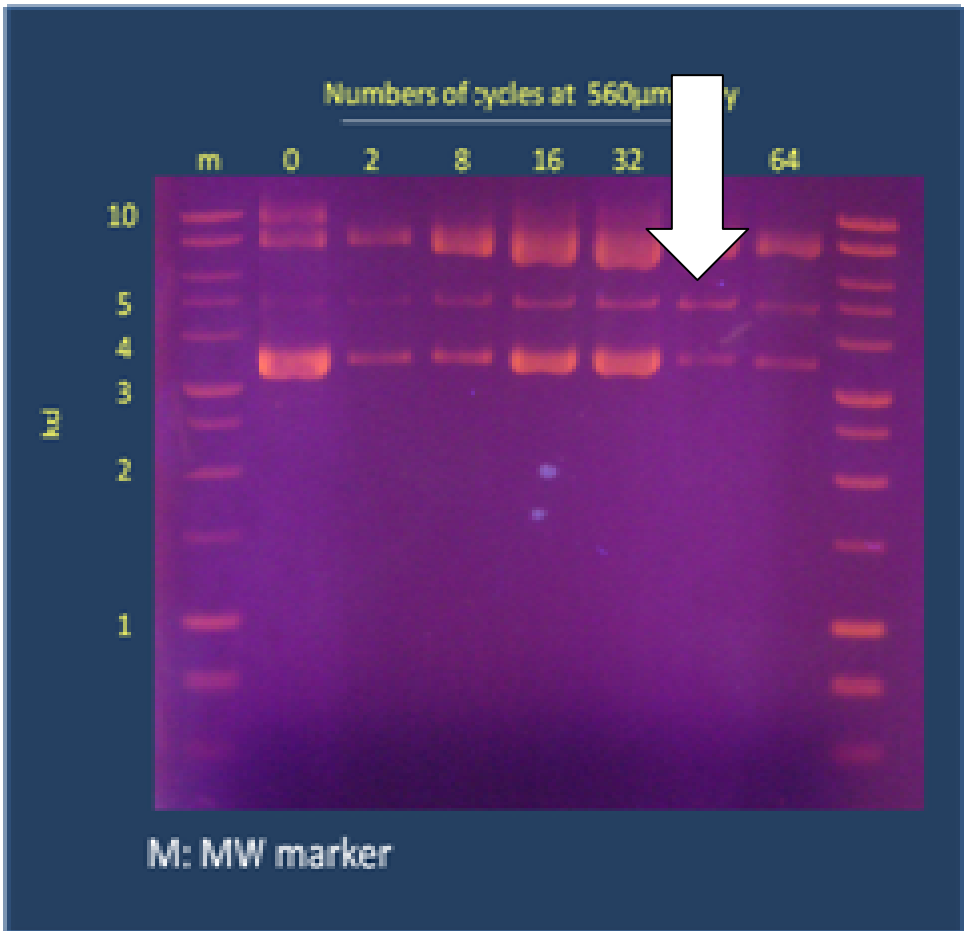


Figure 33. Uncoiling of supercoiled DNA as a consequence of exposure to SR. The band showing appearance of uncoiled DNA is marked with an arrow.

The strain of *Pichia stipitis* that was selected for this research is a high xylose-producing strain originally isolated at LSU. Growth of *P. stipitis* on xylose in the presence of 2-deoxyglucose was found to be inhibited only when the 2-DG concentrations were 2 % w/v or greater (Figure 38). A series of mutation screens were conducted and though numerous mutants were selected, none met the requirements for a biomass utilizing culture This problem did not appear to be with the process of mutation with rather with a lack of specificity in the selection process. Efforts will continue along this line to produce the required mutants. The radiation mutation using SR radiation did increase the number of potential mutants that could be selected for further screening.

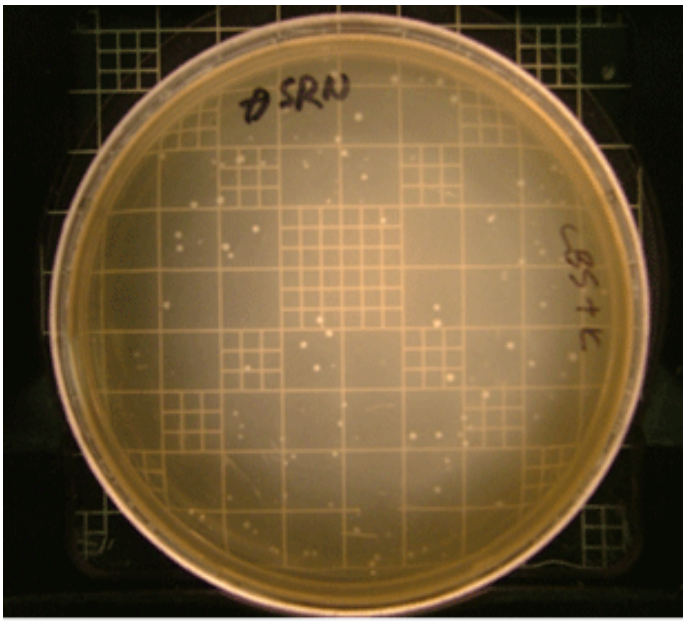


Figure 37. *E. coli* colonies producing dextran (dextransucrase+). The larger colonies are desired mutants, the smaller do not contain the desired gene.

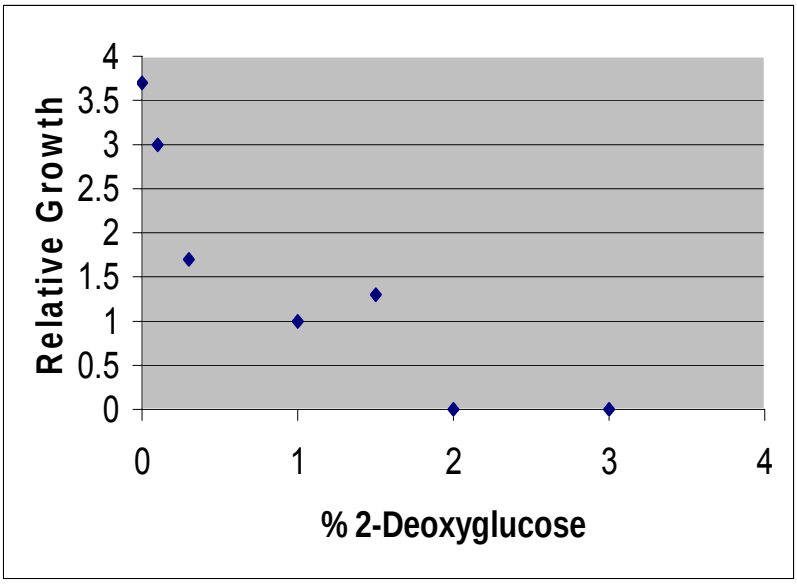
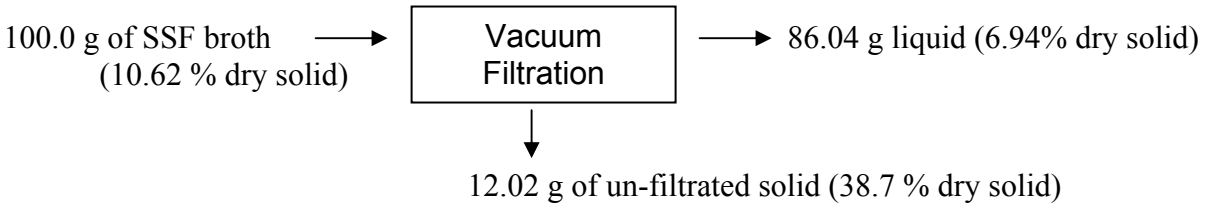


Figure 38: Growth of *P. stipitis* on Yeast Peptone xylose media in presence of 2 - deoxyglucose.

The results indicate that the use of synchrotron radiation is a viable alternative to chemical mutagenesis. Mutations were produced in both dried cells and in isolated DNA samples. Changes were also produced in supercoiled DNA. The supercoiled DNA uncoiled as a result of Synchrotron radiation exposure. SR is a viable tool for the development of mutants of industrial importance. Conditions need to be standardized, including energy and time discrimination as well as reproducibility and dose calculations. Additionally, temperature should be monitored to avoid other scurrilous reactions. Still, the initial results are indeed promising and represent a practical approach using synchrotron radiation for mutagenesis.

Stillage and utilization
This task was focused on identifying any potentially valuable by-products that might exist in the alcohol stillage from dil-ammonia treated biomass, fermented stillage.

A batch fermentations of *Saccharomyces cerevisiae* were prepared from dilute ammonia treated biomass hydrolysate (described elsewhere in this report). The fermentation was conducted in 1L flasks. The solids loading was 10% with biomass. The saccharification at 50 °C and pH 5 and SSF process followed when fermentation temperature was lowered to 30 °C with inoculation of *S. cerevisiae*. After fermentation, the alcohol was removed by distillation and the solid and liquid were separated by vacuum filtration using a filter paper (Whatman #1, Whatman Co., Florham Park, NJ). The mass balance is the following.



The sugar composition of the beer is shown in Table 31.

Table 31. Composition analysis of SSF un-filtered solid.

Component	Concentration (g/ 100g of liquid)
Arabinose	0.13-0.14
Xylose	1.45-1.57
Glucose	0.024- 0.03
Cellobiose	Not detected
Total Phenolic Compounds	0.103 ± 0.04 (as gallic acid)
Ash	9.76
COD	10.2 g/L

Cellobiose was not detected in liquid of SSF fermentation broth. Xylose and arabinose are major monomeric sugars. The liquid part was concentrated 2.5 times using a rotary evaporator to distill off residual ethanol. This concentrate was used to test for effectiveness of anaerobic digestion.

GC-MS analysis was conducted on extracted samples from the above liquid to screen phenolic compounds for potential separation. Because of difficulties of separation of phenolic compounds when the concentration is low, only the compounds higher than 2 area percentage are listed in Table 32. The concentration of vanillin (retention time: 8.425) with ammonium hydroxide pretreatment was lower than 0.2 area percentage.

Table 32. Phenolic composition of the stillage

Retention time (min)	Compound ID from Library	Qual.	Area %	CAS #
4.276	Phenol	91	2.22	000108-95-2
6.421	Benzoic acid	96	41.13	000065-85-0
8.100	4-acetyl-1,5-dimethylpyrazole	83	5.24	021686-05-5
8.718	Ethanone, 1-(2-hydroxyphenyl)-	91	3.80	000118-93-4
8.851	4-hydroxy-3-methoxybenzyl alcohol	98	2.94	000498-00-0
10.868	p-phenylphenol	96	3.78	000092-69-3

The compound in highest quantity was benzoic acid. The market for benzoic acid is currently such that extraction of this material does not seem to make economic sense.

Stillage as substrate for production of methane in an anaerobic digester

The SSF fermentation broth from above was concentrated 2.5 times using a vacuum rotary evaporator to prepare a stillage sample for anaerobic digestion. The stillage samples were mixed at 1:1 (v:v) with d-water. This mixture was diluted 20 times with d-water and used for anaerobic methane production. As seed inoculums, 10 % (w/w, as is, 80 % dry solid) of soil collected from compost soil, to the diluted solution was used. To get true methane production only from stillage, methane production of a control not containing stillage was also monitored. A second control, without stillage was autoclaved twice to sterilize and monitored for the base line of anaerobic digestion. Samples were collected at desired times, and Agilent 6890 Gas Chromatography (Agilent Technologies, Palo Alto, CA) coupled with flame ionization detection (GC-FID) was used for measurement of methane. All tests were in triplicate. The results are shown in is Fig.36.

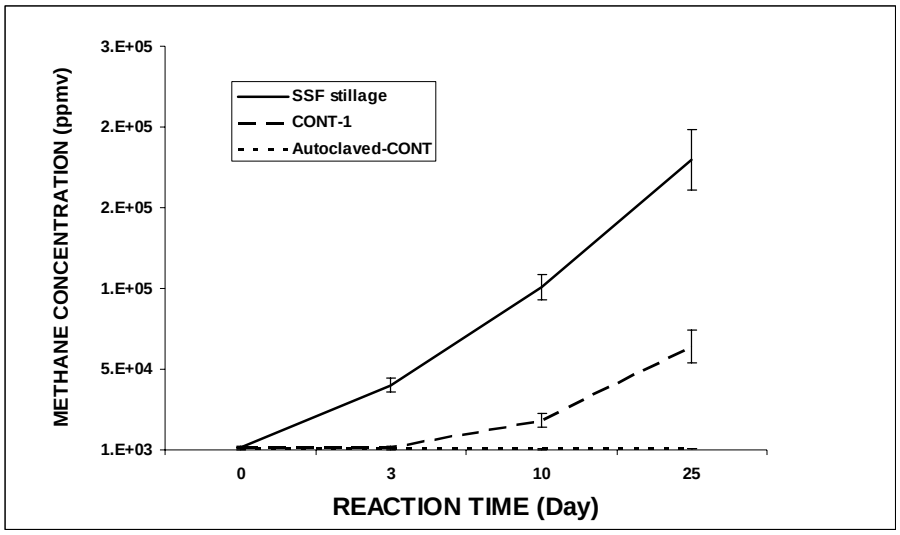


Figure 36. Methane production by anaerobic digestion from biomass generated stillage.

Economic Evaluation

In order to establish a baseline for determining the advantages for converting a raw sugar mill to a biorefinery a model was constructed for financial balance of a Louisiana sugar mill processing 1 million tons of sugar cane in a three month season. This was based on operational data obtained from a Louisiana sugar mill in 2006-7.

SUGAR MILL ECONOMICS				
	Season			**FILTER CAKE**
Tons of sugarcane	1,000,000			Filter Cake, lbs/ton cane
Grinding rate per hour	450.0			Pol % Filter Cake
Grinding rate per day	10,800.0			
Grinding days required	92.59			**BOILING HOUSE**
Lost Time % Total Time	5.01			Brix % Final Molasses
Grinding days (with lost time)	97.23			Purity Final Molasses
				Purity Raw Sugar
Pol%Cane	12.50			Recovery SJM
Fiber%Cane	15.00			Winter Carp Recovery
Brix%Cane	14.71			Boiling House Efficiency
TRS, lbs 96 Sugar/ton cane	209.12			Recovery
				Yield Sugar, 96 M&E%Cane
Brix % Diluted Juice	13.74			CRS, lbs 96 Sugar/ton cane
Purity Diluted Juice	85.92			Liquidation Factor
Mixed Juice % Cane	95.43			Final Molasses % Cane
Imbibition % Cane	30.00			Gal/ton cane @ 79.50
Imbibition % Fiber	200.00			
Pol % Bagasse	3.57			
Moisture % Bagasse	52.00			**POL ACCOUNT**
Fiber % Bagasse	43.39			In bagasse
Bagasse % Cane	34.57			In Final Molasses
Last roll Purity	77.45			In Filter Cake
Brix % Bagasse	4.61			Undetermined Losses
				Total Losses
EXTRACTION				Recovered in Sugar
Pol % Pol in Cane	90.12			In Juice
Reduced (F=12.5)	92.00			In Cane
Abs. Juice lost in bag% Fiber	56.00			
Corrected Reduced Extraction (pr)	89.97			
EVAPORATION				**PROCESS SUPPLIES**
Brix % Syrup	65.00			Gas, mcf/tc
Purity % Syrup	85.92			Lime, lbs/tc
Water Evaporated, tons/hr	338.65			HCl Acid, gals/tc
				Caustic Soda, gals/tc
				Flocculant, lbs/tc
				Dextranase. lbs/tc
				Amylase, lbs/tc

SUGAR MILL COST MODEL					
Grower shares					
Sugar	60%				
Molasses	50%				
Market Prices					
Sugar ($ per lb)	$ 0.200				
Molasses ($ per gal)	$ 0.500				
Sugarcane freight ($/ ton Cane)	$ 3.50				
Sugar freight, other ($ ton Cane)	$ 2.00				
FACTORY EXPENSES					
Grinding cost per day	$ 38,000				
Offseason expenses					
Employee expenses					
Administrative expenses					
Depreciation expenses					
TOTAL EXPENSES					
SALES					
SUGAR (lbs)	217,211,416				
MOLASSES (gals)	7,501,922				
TOTAL SALES	$47,193,244.00				
COST OF SALES					
CANE PURCHASES	$27940850.00				
SUGARCANE FREIGHT	$3,500,000.00				
SUGAR FREIGHT	$ 217,211.00				
TOTAL COST OF SALES	**$31,658,061**				
GROSS PROFIT = SALES (Sugar, Molasses) - COST OF SALES					
				$15,535,183.00	
NET INCOME FROM FACTORY OPERATION = GROSS PROFIT - FACTORY EXPENSES					
				$12,039,183.00	

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APPENDICES

Analytical methods

HPLC Analysis

Sugars from Composition Analysis. Glucose, cellobiose, mannose, arabinose and xylose were analysed using an Aminex® HPX-87P, 300 mm X 7.8 mm column. Samples were run for 22 min in water.

Fermentable Sugars and alcohol. Glucose, cellobiose, mannose, arabinose, xylose and ethanol were analysed using an Aminex® HPX-87K, 300 mm X 7.8 mm column. Samples were run for 25 min in 0.01 M K_2SO_4 solution.

Analysis of the composition of sugar cane bagasse and pretreated bagasse

The carbohydrate composition and the content of ethanol extractives, ash and lignin were determined using the NREL laboratory analytical procedures (www1.energy.gov/biomass/analytical_procedures.html). The moisture content of the bagasse samples was determined using a moisture analyzer (Computrac MAX 1000, Arizona Instrument Corporation, Tempe, Arizona).

Enzyme Loading**:**
Spezyme CP30 FPU/g glucan; Novozyme 188
15 CBU/g glucan

Composition Analysis post SSF**:**

Sample***	Percent (g/ 100 g dry mass)							
	Ash	Extractives	ASL*	AIL**	Glucose	Xylose	Arabinose	Mannose
Biomass	19.81	14.57	4.31	36.52	15.41	5.85	0.26	1.28
NIST Standard†	3.95	1.37	4.34	19.33	41.09	23.98	1.40	0.10
NIST Range‡	4.0 ± 0.5	4.4 ± 5.3	2.0 ± 2.0	22.3 ± 2.5	40.2 ± 3.2	21.5 ± 3.1	1.8 ± 0.5	0.4 ± 0.4

*= Acid soluble lignin; **= Acid insoluble lignin; ***= Biomass at 10% solids loading; †= NIST Standard run as internal control; ‡NIST Range, Reference values provided by the National Institute of Standards & Technology.

Pretreatment of sugar cane bagasse

Bagasse from sugar cane (*Saccharum officinarum*) was obtained from the Raceland Raw Sugar Corp. sugar mill, Raceland, Louisiana and ground to a particle size less than a 12 mm. Ground sugar cane bagasse has submitted to MBI International, Lansing, MI for pretreatment using the AFEX process (Holtzapple et al., 1991). Briefly, approximately 1 kg of the bagasse was treated in a one gallon reactor at 100 °C for 30 min with a 2:1 ammonia loading to biomass and 40 % moisture level. After pretreatment, bagasse was removed from the reactor, dried to remove ammonia and stored in sealed plastic bags at 4°C. Ground sugar cane bagasse (2.5 kg) was also pretreated by adding a 28 % stock solution of ammonium hydroxide to achieve a final concentration of 0.02 g NH_3 /g water in a final water mass of 20 kg. The slurry was placed in a pressure reactor and heated at 160 °C for 60 min. The solid mass was removed from the slurry by filtration through muslin cloth and washed with 40 kg of water. Less than 1 % of mono sugars in the bagasse were removed by this pretreatment.

Pretreatment Studies- Pre-treated biomass from studies conducted with or without addition of make-up ammonia was evaluated for ethanol production by SHF and SSF. Both SHF and SSF studies were

conducted for 72 h. SHF studies were carried out in duplicates at 10% solids loading (10 g pretreated bagasse/100 g total mass) without the supplementation of molasses. Novozyme 188 and Spezyme CP were the enzymes used during saccharification at 55°C for 24h. *Saccharomyces cerevisiae* was added for the conversion of glucose to ethanol at a final concentration of 1 X 10^7 CFU/ml. SSF studies were conducted at 33°C for 48 h. Ethanol concentrations were analyzed by GC. Sugars and organic acids were analyzed by HPLC.

Pretreatment conditions -Unwashed sugarcane bagasse was pretreated using dilute ammonia at 160°C for 1 h. The liquid stream was recovered and recycled twice for a total of three runs (Figure 1). Biomass, water and ammonia added as ammonium hydroxide (stock solution: 29%v/v) were loaded into the reactor to a final ratio of approximately 1:8:0.14, respectively.

Chemical Pretreatment - Approximately 20 g of dried bagasse were treated with 50% (w/w) ethanol, 5% chemical NaOH, 10% chemical Ox-B (a sodium hypochlorite and hydrogen peroxide solution) or combinations of 5% chemical NaOH/50% ethanol and 10% Ox-B/10% ethanol. Each bagasse sample was boiled for 2 h at atmospheric pressure. The pre-treated bagasse was filtered by vacuum through a filter, and the liquid portion collected for analysis of total phenols. The solid portion was collected and pre-treated sequentially with 10% ethanol, 5% chemical NaOH, 10% chemical Ox-B or combinations. The last sequential treatment involved treatments with only 15% chemical NaOH. Total phenol compounds (TPC) were determined for each pre-treatment and sequential treatment. Figure 1 summarizes the pre-treatments and sequential treatments of sugarcane bagasse performed in this study.

Batch hydrolysis of pretreated sugar cane bagasse and Avicel

Enzymatic saccharification experiments of pretreated samples were performed in triplicate in 20 ml glass scintillation vials at 50 °C and 100 rpm? for 72 h as described in the NREL (LAP-009) procedure. Briefly, the reaction mixture contained 0.1 g cellulose (dry weight) (except with the substrate loading experiment), 0.5 ml 1 M sodium citrate buffer (pH 4.8), 40 µl tetracycline (10 mg/ml), 30 µl cycloheximide (10 mg/ml), Spezyme CP and Novozym 188 in a ratio of 1:2 and distilled water to give a final volume of 10 ml. Adjustments were also made for the addition of various activities of commercial and laboratory-produced xylanases. The moisture content in the AFEX-pretreated bagasse (24.53 %) and NH_4OH-pretreated bagasse (78.42 %) was included in the calculation of the total volume. Avicel (FMC Biopolymer pH-102; 4.54 % moisture) was also included as a control. Substrate blanks excluded the enzyme activities whereas enzyme blanks excluded the substrates and the degree of hydrolysis was used to correct data. Samples were withdrawn initially and after 72 h and centrifuge at 10,000 rpm in Eppendorf tubes to remove the biomass. Subsequently the liquid portion was filtered through 0.45 µm (pore size) filters (25mm (diameter) Whatman GD/X filter (PTFE filter media with polypropylene housing, Cat. No. 6874-2504, Whatman Inc. Florham Park, NJ) and the liquid subjected to HPLC and reducing sugar analysis. HPLC analysis was conducted using an isocratic HPLC system using a refractive index detector (Spectra System. RI 150, Thermo Electron Corp., Milan, Italy). Sugars were separated on a BioRad Aminex-HPX-87P column (BioRad Lab. Inc. Hercules, CA) at a flow rate of the mobile phase (DI water) of 0.6 ml/min and 85°C for 30 min. Reducing sugar concentration was determined as described above. Generally the reducing sugar concentrations agreed within 10 % of the total sugar determined by HPLC. In instances where the values did not agree, analysis was repeated.

Calculations - The percent hydrolysis of the glucan fraction of sugar cane bagasse was calculated adding the glucose and double the cellobiose concentrations, correcting for the hydration (each glucose molecule had one molecule of water added during hydrolysis and therefore the glucose concentration

was multiplied by 0.9) and dividing by the grams of glucan. Similarly, the percent hydrolysis of the xylan fraction was calculated by adding the xylose and arabinose concentrations, correcting for hydration (total xylose and arabinose was multiplied by 0.88) and dividing by the grams of total xylan and arabinan.

Enzyme Activities.

Glucan synthase activity - For the determination of GS activity, 0.7 mM UDP-G was reacted with 0.83 µg/µl GS in 27 mM HEPES (pH 7.2) containing 7 µM GTP, 1.3 mM EDTA, 0.17% Brij 35, and 2.2% glycerol in the addition of 0, 0.12, 0.24, 0.36, and 0.48 g/ml CBO at 22°C for 105 min. A Sirofluor™ binding with 1,3-β-$_D$-glucans was then conducted as described in the material and method section. The fluorescence was measured excitation wavelength of 390 nm and emission wavelength of 455 nm. Error bars show the standard deviation error of the mean.

Commercial preparations - A commercial preparation produced by *Trichoderma reesei* (Spezyme CP) and supplied by Genencor (Rochester, NY) was used as the main cellulase enzyme in this study. This preparation was supplemented with a β-glucosidase preparation (Novozym 188) produced *Aspergillus niger* and supplied by Sigma (C6105). Various commercial enzymes with cellulose and xylanase activity were kindly provided by various North American suppliers (Table 1) and their cellulolytic and hemicellulolytic activities were evaluated (Tables 3-4). Some enzyme samples were in powder form and were suspended in 100 mM sodium acetate buffer (pH 5) at a concentration of 10 mg/ml.

Laboratory-produced enzyme preparations - *Thermomyces lanuginosus* strains SSBP and ATCC 34626 and *Aspergillus carneus* Abo 372 were obtained from the culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa. The fungi were maintained on malt extract agar plates. With a sterilized needle, samples were inoculated into 1 ml YPD (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose), and cultivated for 2 days respectively at the optimum temperature for the fungus (*T. lanuginosus*, 50°C; *A. carneus,* 30°C) with shaking. The contents of each inoculant were decanted into 200 ml growth medium (0.67% Yeast Nitrogen Base, with amino acids, 0.2% L-Asparagine monohydrate, 0.5% KH_2PO_4), containing 4 g of AFEX pretreated sugar cane bagase or 4 g beechwood xylan (Sigma X 4252) as carbon source and cultivated for 5 days at the optimum temperature. Fungal growth was clearly visible in the flasks after 4 days. The culture fluid was filtered through 4 layers of muslin cloth and then centrifuged at 5 000 X g for 10 min to remove fungal debris. After centrifugation, 0.1 % sodium azide was added to the supernatant and concentrated by filtration through an Amicon concentration apparatus using a 10 kDa membrane filter.

Enzyme and protein assays - The filter paper activity of enzyme samples was determined at 50 °C according to standardized NREL filter paper assay (Adney & Baker, 1996). Carboxymethylcellulase (CMCase; endoglucanase), avicelase (exoglucanase) and β-D-(1,3;1,4)-glucanase activity was determined by measuring the release of reducing sugars from respectively 3 % carboxymethyl cellulose (Sigma), 3 % Avicel (FMC Biopolymer pH-102) and 0.5 % barley β-glucan (Sigma) at 50 °C and pH 5.0 (100 mM acetate buffer) for 10 min (Wood and Bhat, 1988). The reaction was terminated by addition of dinitrosalicyclic acid reagent and subsequently boiled for 5 min. The reducing sugar concentration was determined from the absorbance at 540 nm using a glucose standard curve as reference. One unit (IU) of activity was defined as the amount of enzyme that released 1 µmol of glucose as reducing sugar equivalents per minute.

β-Xylanase activity was determined by following the release of reducing sugars from a 1.0% birchwood xylan (Sigma) solution at 50°C for 5 min (Bailey *et al*., 1992). The reaction was terminated by addition of dinitrosalicyclic acid reagent and subsequently boiled for 5 min. The reducing sugar concentration was determined spectrophotometrically at 540 nm from a xylose standard curve. One unit of activity was defined as the amount of enzyme that released 1 μmol of xylose as reducing sugar equivalents per minute.

Cellobiohydrolase (CBHI), β-glucosidase, β-xylosidase and α-arabinofuranosidase activities were determined by following the release of 4-nitrophenol from respectively 4-nitrophenyl-β-D-lactopyranoside, 4-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl-β-D-xylopyranoside and 4-nitrophenyl-α-L-arabinopyranoside (Sigma) for 15 min at 50° C and pH 4.0 (50 mM Na acetate buffer). The reaction was stopped by the addition of 1 M Na_2CO_3 and absorbance read at 410 nm from a 4-nitophenol standard curve. One unit of activity was defined as the amount of enzyme that released 1 μmol of 4-nitrophenol per minute.

The protein concentration was determined using the Coomassie Brilliant Blue dye-binding method (Bio-Rad; Bradford, 1976).

Lignin analysis

Colorimetric Evaluation of Total Phenols - The total phenols were determined colorimetrically at 765 nm using the Folin-Ciocalteu reagent and are expressed as mg vanillin g-1dried bagasse.

Characterization of Total Phenols by GC/MS - Approximately 20 ml of liquor, collected after each pre and sequential treatment of sugarcane bagasse, was extracted three times with equal volumes of dichloromethane. The extracted samples were evaluated using a system Varian CP 3800, Saturn 2200 GC/MS. Sample separation was made using a WCOT fused silica capillary column, CP 8CD, 30 m X 0.25 mm i.d. with 0.5 μm film thickness. The GC column oven temperature was set to 280°C. Compounds were tentatively identified using NIST Library.

Organisms and Preparation

Pichia stipitis was obtained from Dr. Meredith Blackwell of the Dept. of Plant Pathology at LSU. It was isolated originally from the gut of a wood-ingesting beetle (*Odontotaenius disjunctus*). The culture was routinely maintained on YM (Difco) agar slants, and propagated in YM broth at 30°C (Van Zyl et al. 1988).

Leuconostoc mesenteroides B-512FMC was used as a control system. The organism was grown on LM media as described by Kim et al., 1997. *Escherichia coli* B containing a pUC plasmid with kanamycin resistance was used as a host strain.

DNA Preparation - DNA from *L. mesenteroides* was isolated according to the procedures recommended by Qiagen (Valentia, Calif.) for their QIAprep Spin Miniprep kit as reported by Kang et al. 2005. Irradiated DNA was ligated to the plasmid pGEM-3Zf(-1) and transformed into *E. coli*.

X-ray exposure - Because of the necessity for direct exposure of the samples to the synchrotron beam, dried cultures were utilized to withstand the vacuum requirement.. A special test chamber (chip) was designed and constructed by CAMD for this research (Figure 1). Initially, the chips were constructed of polymethyl methacylate. This material was found to deform on autoclaving and the charge on the

polymer resulted in poor recovery of both exposed and unexposed cells. A second, acceptable, version was cast from polycarbonate. A micromachining beamline equipped with a Jenoptik scanner was used to expose eukaryotic and prokaryotic cells and isolated DNA, in a dried state, to a specific X-ray spectrum and damage to DNA was monitored using genetic manipulation techniques.

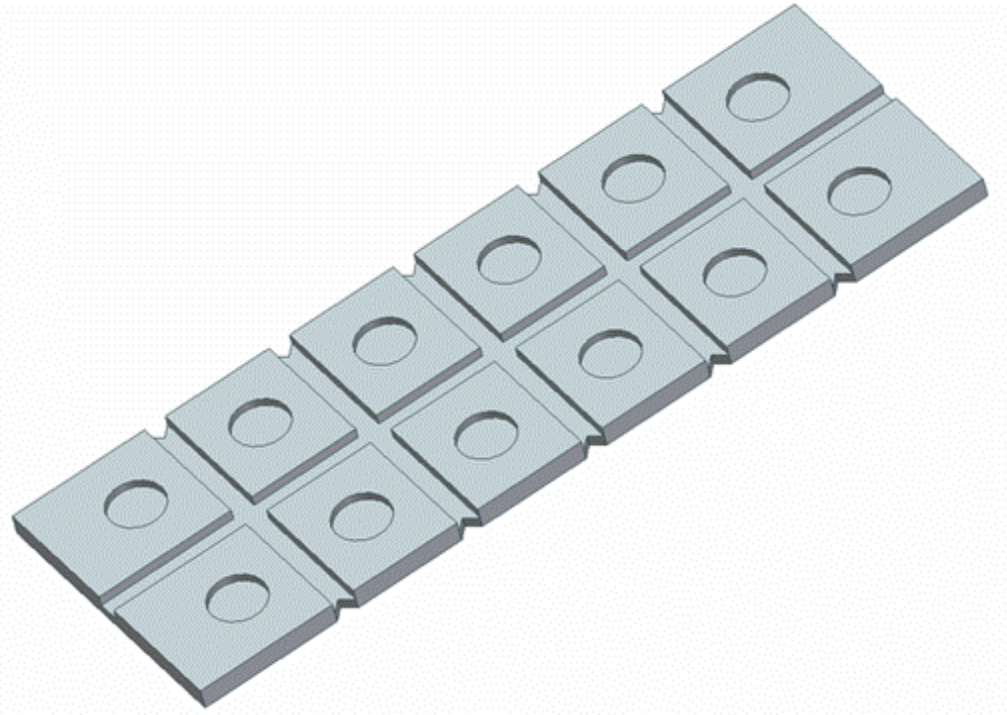

Figure 1: Chip for X-Ray mutation research. Microscope slide-sized exposure tool. The tool is molded in polycarbonate, then cut into final shape with a bandsaw. Twelve holes, each with a capacity of 10 microliters, permited quadruple replicates of each of 3 samples for each exposure condition.

Extracted DNA or 10 µl aliquots of Pichia cells of known concentrations were placed in a specially designed, microfabricated holder. The Pichia was grown in liquid media at 30 °C, with shaking at 200 rpm for 24 h. Absorbance readings at 600 nm were converted to direct counts. Samples were air dried prior to x-ray exposure. For isolated DNA, know aliquots were dried onto wells in the exposure plate. After exposure they were removed with a saline solution and transduced into *Escherichia coli* B. Transduction was monitored by detecting dextransucrase production due to changes in colony shape when the cultures were grown on a sucrose containing media. Dextransucrase producing cells produce large, slimy looking colonies.

Mutation Screening - Pichia was screened for mutants by growth on xylose in the presence of 2-deoxyglucose, a non-metabolizable glucan which simulates glucose. The goal is to create a strain which will simultaneously utilize xylose and glucose. The amount of 2-deoxygluxcose required to completely repress growth on *P. stipitis* on xylose, in the presence of glucose was found to be 2% (Figure 2). Screening was conducted on 1.5% 2-deoxygluxcose and colony size used as a marker for mutation. Transductants in *E. coli* were detected by colony growth on sucrose containing nutrient agar plates. DNA changes were determined directly using agarose gel electrophoresis with ethidium bromide staining.

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