Improvements in SSF technology for ethanol production from sugarcane bagasse

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Presented to IEA Bioenergy
Task 39 - Session 2C
UBC Vancouver, Canada
29 August 2006

Celunol

Key Barriers to Lignocellulosic Ethanol

- Feedstock Cost
  - Near-term cost will limit size of plants
- Enzyme cost
  - DOE-funded cost reduction is still not a reality
- C_5/C_6 fermentation
  - SHF vs. SSF
- Risk
  - Cost of the first plant(s)
    - Capital Cost and Cost of Capital

Celunol Licensed Technology

Addressing the barriers

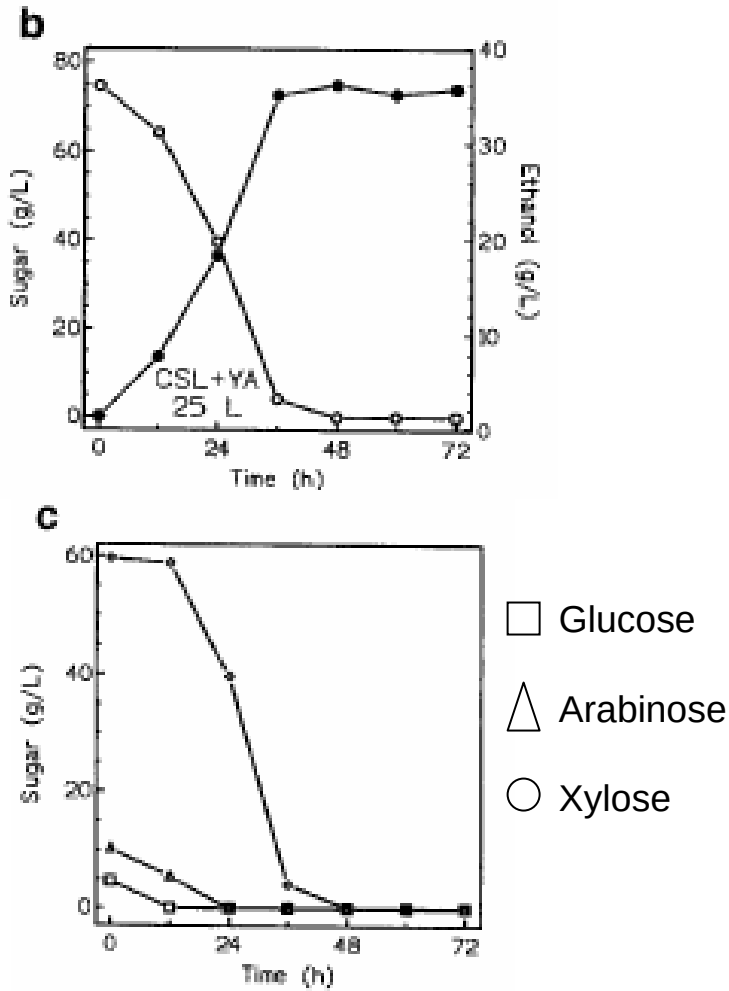
- Feedstock cost
  - Start with lower cost agriculture residue
- C_5/C_6 fermentation increases yield
  - Pentose/hexose co-fermentation
- Enzyme cost
  - Enzyme Co-production
  - In-house enzyme production
- Risk
  - Seeking Federal Funding and Loan Guarantees

Escherichia coli

Engineered for ethanol production

- University of Florida
- Robust
- Ferments mixed sugars
- High yields
- High rates
- Low nutrients
- Industrial history
- BSL 1 Safe

Fermentation of acid hydrolysate of sugarcane bagasse at 25 liter scale



Asghari, et.al. 1996 JIM 16:42

Robust - to process errors

Effects of process errors on the production of ethanol by Escherichia coli KO11

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Escherichia coli KO11 was previously constructed for the production of ethanol from both hexose and pentose sugars in hemicellulose hydrolysates by inserting the Zymomonas mobilis genes encoding pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adhB). This biocatalyst appears relatively resistant to potential process errors during fermentation. Antibiotics were not required to maintain the maximum catabolic activity of KO11 even after deliberate contamination with up to 10% soil. Fermentations exposed to extremes of temperature (2 h at 5°C or 50°C) or pH (2 h at pH 3 or pH 10) recovered after re-adjustment to optimal fermentation conditions (35°C, pH6) although longer times were required for completion in most cases. Ethanol yields were not altered by exposure to extremes in temperature but were reduced by exposure to extremes in pH. Re-inoculation with 5% (by volume) from control fermentors reduced this delay after exposure to pH extremes.

Journal of Industrial Microbiology & Biotechnology (1998) 20, 281–286

Process Error recovery

Table 1 Effect of simulated process errors on cell growth and ethanol production[a]

Treatment	Maximum cell density[b] (gdw L^{-1})	Base added[c] (mmoles L^{-1})	Maximum ethanol[d]		Ethanol yield[e] (% theoretical)
			Time (h)	Conc. (g L^{-1})	
None (n = 14) control	3.3 ± 0.2	45.7 ± 8.2	48	42.6 ± 1.9	93 ± 4
50°C, 2 h	2.9	65.7	60	39.3	89
+ 5% inoculum	2.7	62.9	60	39.5	89
5°C, 2 h	3.6	57.4	60	40.8	91
+ 5% inoculum	3.2	48.6	60	41.2	92
pH 10, 2 h	3.6	97.1	96	34.4	79
+ 5% inoculum	4.0	103	48	37.5	86
pH 3, 2 h	3.6	117	96	36.0	83
+ 5% inoculum	3.7	109	72	36.9	85
1 gdw soil L^{-1}	nd[f]	47.1	48	44.3	99
10 gdw soil L^{-1}	nd	42.9	48	43.7	97
50 gdw soil L^{-1}	nd	38.6	48	42.3	94
100 gdw soil L^{-1}	nd	40.2	48	41.2	92
Hydrolysate[g]	nd	8.6	96	41.0	92

[a]Results represent an average of two or more fermentations with 90 g xylose L^{-1}. Control values are averages of 14 fermentations with standard deviations (12 fermentations for cell mass).
[b]Maximum cell density in grams (dry weight) per liter.
[c]Base (2 N KOH) added to maintain fermentation at pH 6 or above.
[d]Values represent total ethanol per liter of original fermentation broth and have been adjusted for dilution by added base.
[e]The theoretical yield from 90 g xylose is 45.9 g of ethanol.
[f]nd, not determined.
[g]Hydrolysate and nutrients were not sterilized. The maximum theoretical yield from 86.9 g sugar is 44.3 g ethanol.

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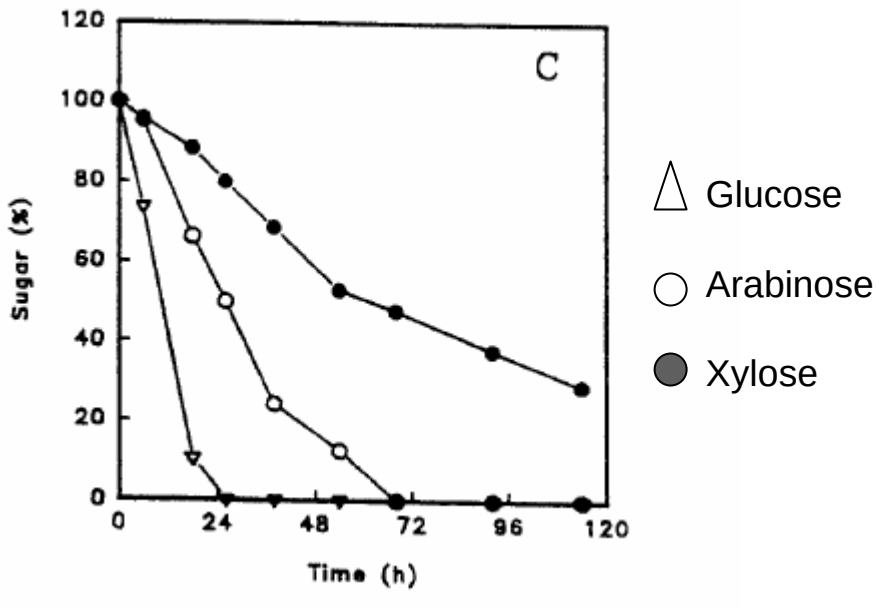
Klebsiella oxytoca

Engineered for ethanol production

- University of Florida
- Ferments mixed sugars
- Low nutrients
- Co-produces enzymes
- pH 5.0 - 5.5
- Utilizes cellobiose and cellotriose



Bothast, etal. 1994 BioTechLett 16:401-406

Development of SSF microbe

- *K. oxytoca* M5A1
  - Natural ability to utilize all five biomass sugars.
  - Natural ability to transport/metabolize dimers and trimers (Glu, Xyl).
- *K. oxytoca* P2 (B.Wood)
  - Chromosomally integrated *pdc* and *adhB* from *Zymomonas mobilis*.
- *K. oxytoca SZ21pCPP2006* (S.Zhou)
  - Chromosomally integrated *celY* and *celZ* from *Erwinia chrysanthemi*.
  - Additional genes for secretion of endoglucanase activity located on the plasmid.
- K. oxytoca BW34 (B.Wood)
  - Expresses celY without secretion system
  - Improved C_5/C_6 co-fermentation

Klebsiella oxytoca

SSF Process

- Co-production of enzymes
- Co-utilization of sugars (when C6 is low)
- Reduces enzyme dosage

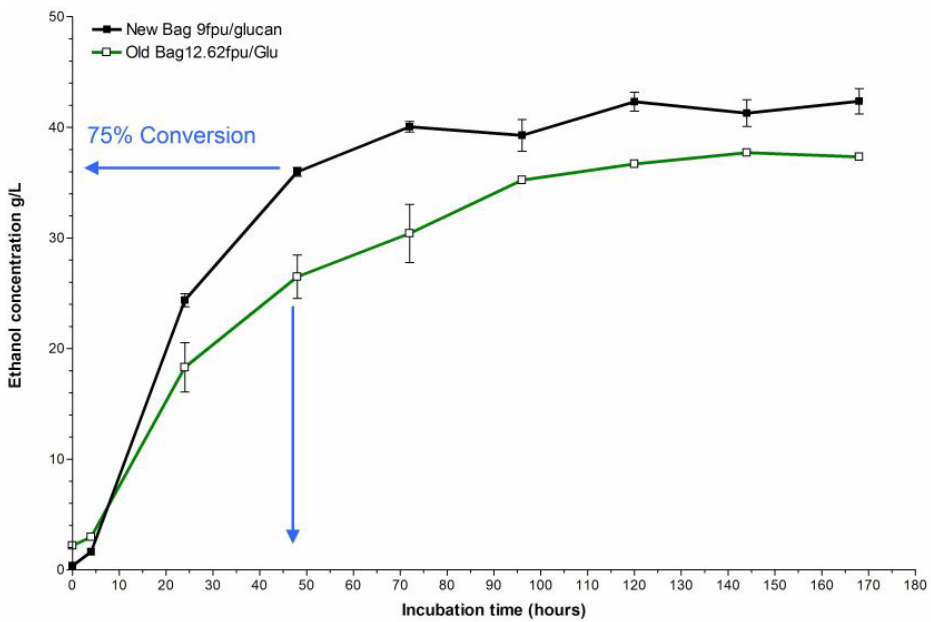


TABLE 4. Effect of *out* genes (pCPP2006) on endoglucanase production by derivatives of *K. oxytoca* P2

Strain or Spezyme additive	CMC zone[a] (mm)	OD_{550}[b]	CMCase activity[c]		
			Extracellular (IU/liter)	Total (IU/liter)	Secretion (%)
P2	0	10.5	0	0	0
SZ6	8.5	11.0	1,920	8,800	22
SZ21	6.7	11.0	1,620	7,800	21
SZ22	2.0	10.0	480	879	55
P2(pCPP2006)	0	10.0	0	0	0
SZ6(pCPP2006)	10.8	9.6	13,800	22,300	62
SZ21(pCPP2006)	11.5	10.2	20,100	26,900	75
SZ22(pCPP2006)	2.0	9.7	449	833	54
Spezyme CE (10 ml/liter)[d]				27,000	
Spezyme CP (10 ml/liter)[d]				33,400	

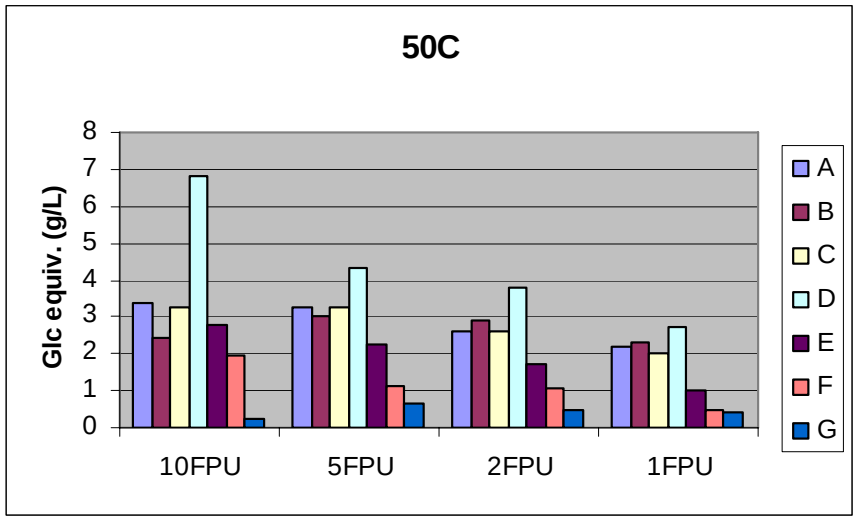
[a] Diameter of cleared zone on CMC indicator plates.
[b] Culture density after 24 h of incubation (30°C in LB medium containing 5% sorbitol).
[c] Endoglucanase activity was measured using cultures grown for 24 h.
[d] Dilution equivalent to the highest Spezyme level used in fermentation experiments (Table 5).

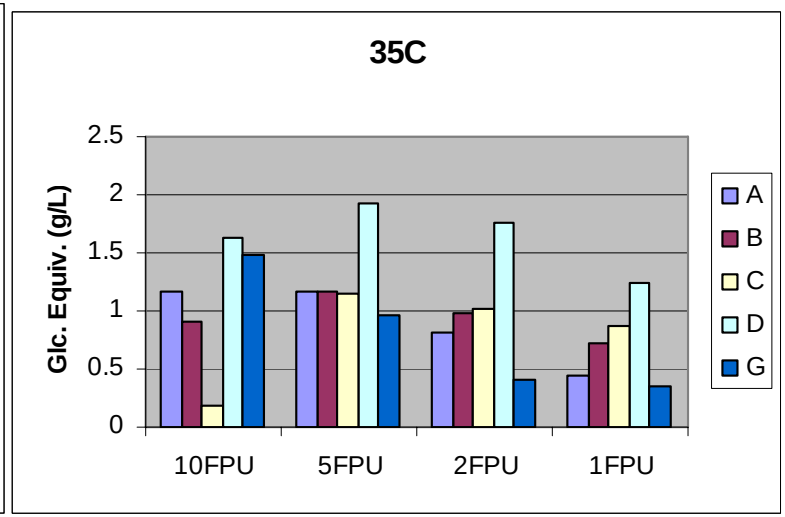
Enzyme Selection

- Enzymatic hydrolysis under standard conditions would lead to choose a different enzyme versus in combination with a co-producing microbe.
- Test for compatibility with *K. oxytoca* BW34 lead us in another direction.

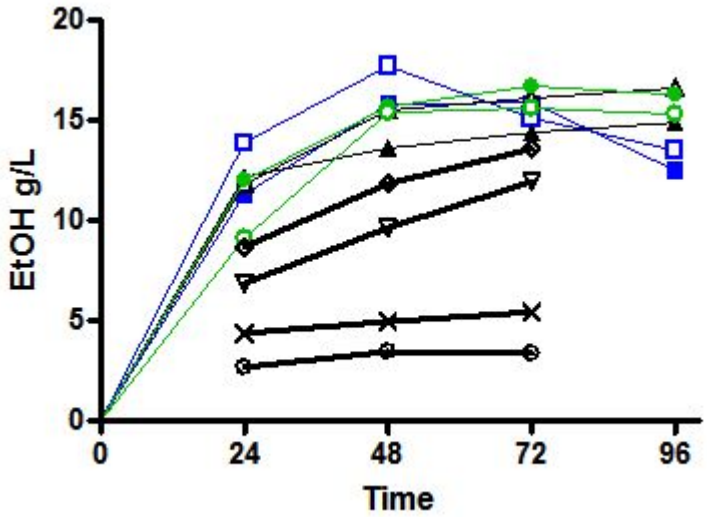
Enzyme hydroysis of bagasse

Survey of enzymes





SSF of bagasse with BW34



A - 5 FPU/g

B

C

D - 10 FPU/g

E

Enzyme Production

- At-site or near-site production for economic viability
  - Eliminate downstream processing and product stabilizers (inhibitory).
  - Improved performance
- Produce enzymes on biomass substrate to optimize

In-house production

Substrate dependence

Source of inducer influences the mixture of enzyme produced.

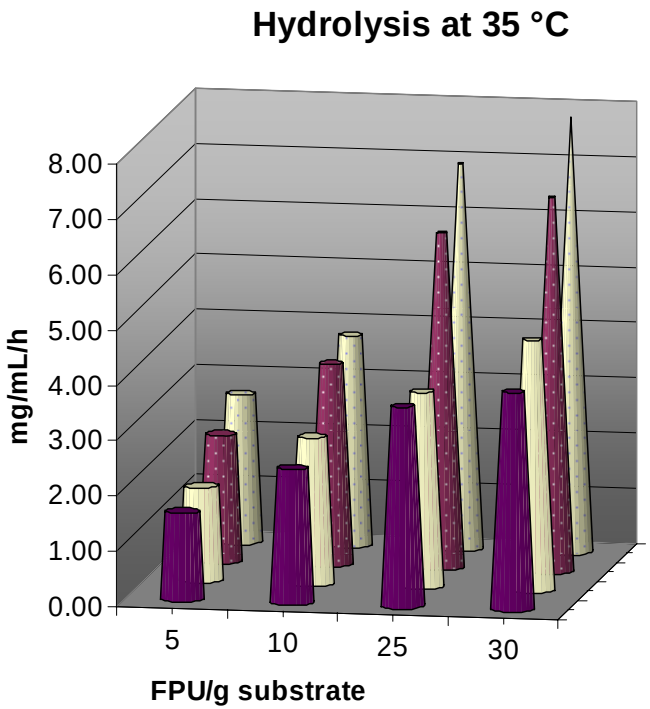
Table 2
Specific enzyme activities and protein concentrations after 7 days of fermentation (R.S.D. of specific activities were below 10%)

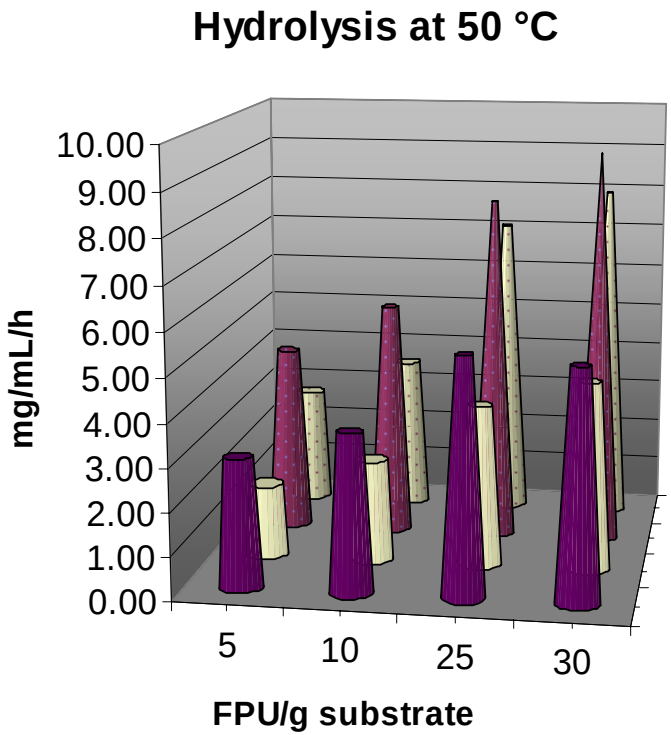
Enzymes	Protein (mg/mL)	Specific enzyme activities (FPU/mg, nkat/mg)										
		FPA	Endoglu-canase	EG I	CBH I	β-Gluco-sidase	Xylanase	Mannanase	Acetyl xylan esterase	α-Galacto-sidase	β-Xylo-sidase	α-Arabino-sidase
SF	1.89	0.58	149	4.2	18.3	8.0	1985	75.0	5.5	0.2	12.5	18.4
CO	2.23	0.52	117	3.8	17.5	4.7	1077	40.5	1.5	1.4	3.5	16.2
SP	0.83	0.45	104	3.7	22.5	8.8	203	33.1	0.4	1.1	2.6	23.5
WI	1.05	0.56	136	5.4	23.0	5.8	868	67.0	2.2	1.3	3.5	23.0
CE	125	0.55	130	4.6	17.1	4.2	100	26.1	14.1	0.1	2.3	4.3
EC	110	0.63	153	6.7	16.3	4.5	255	21.9	7.7	6.4	2.9	13.8

Tengborg, Galbe, Zacchi. 2001 Biotechnol. Prog. 17:110

In-house Enzyme Production

Substrate dependence





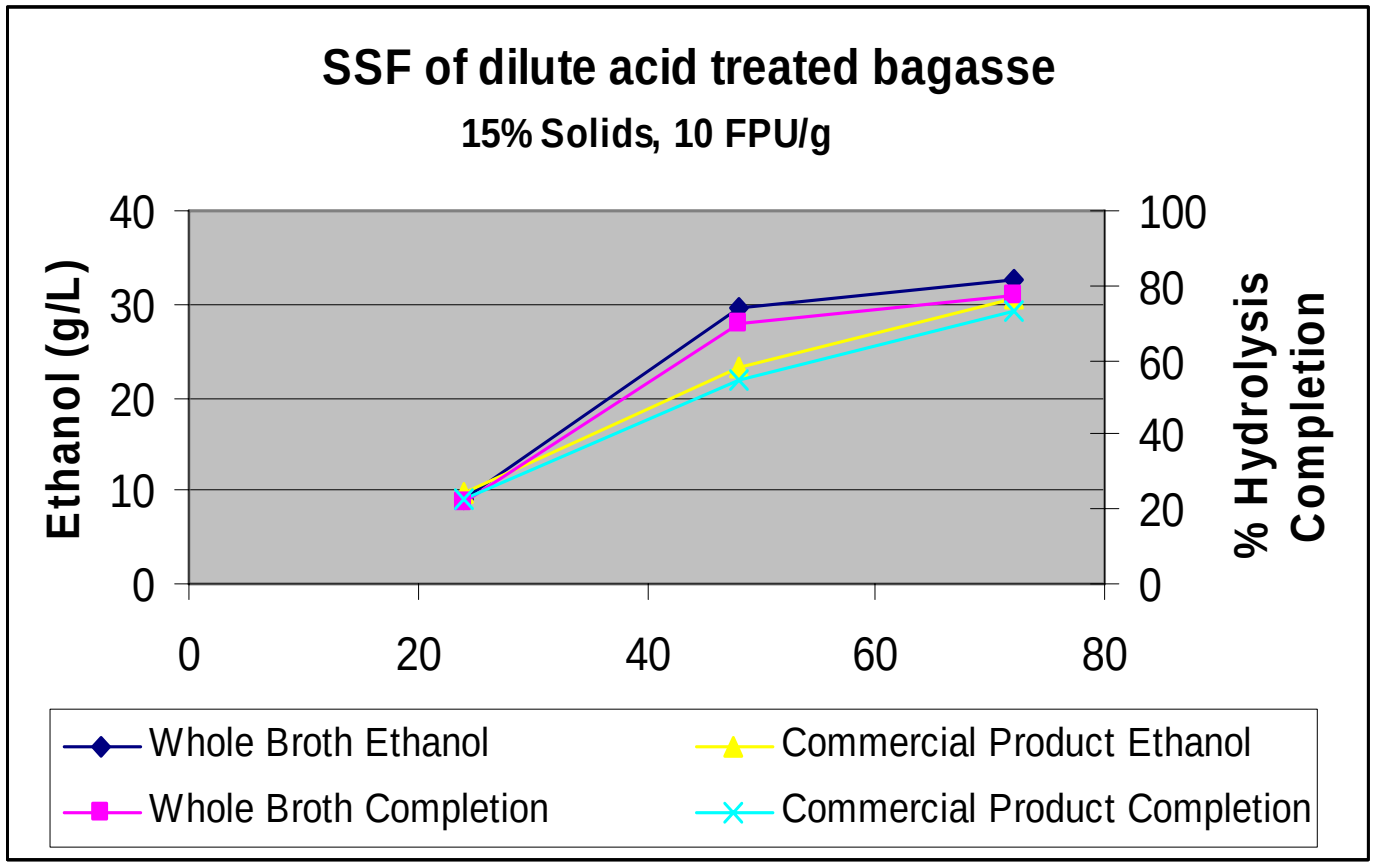
Dotted: in-house produced. Solid – Commercial product

Dilute acid pretreated rice straw (red) and bagasse (yellow)

Luli, Wood and Lee. 2001 Unpublished

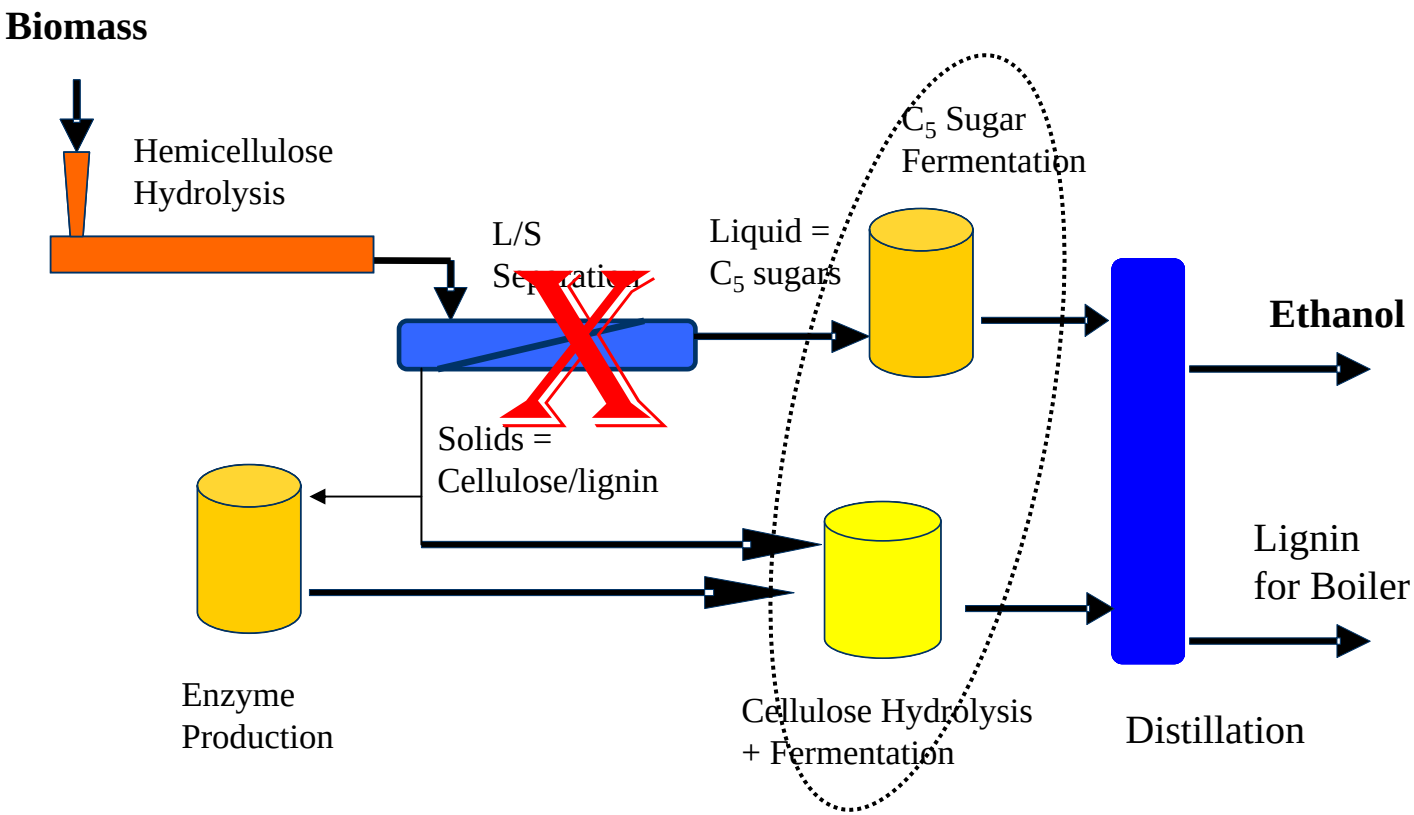
In-house Enzyme Production

Improved performance



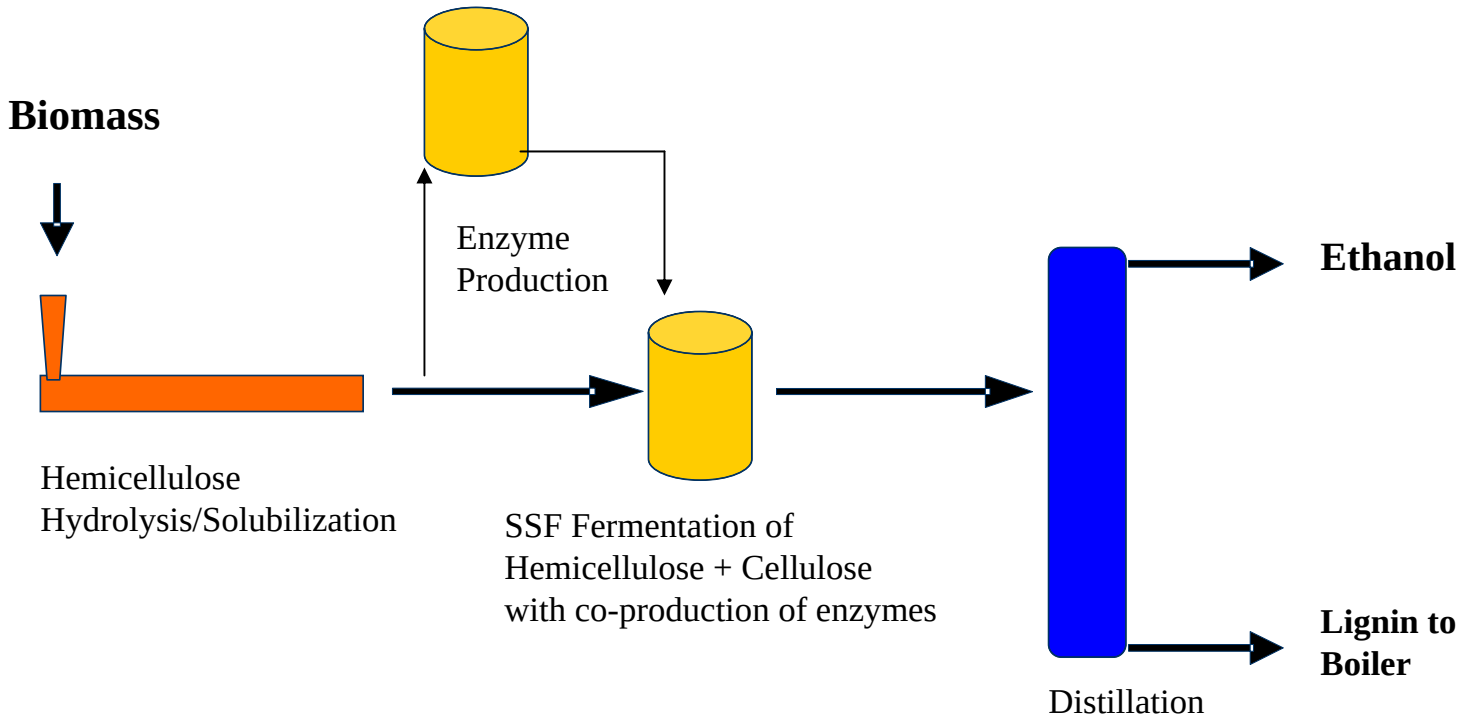
Celunol Process Design

SSF Model



Celunol Technology

Future Process: One Fermentation



Celunol - Path Forward

- Pilot Plant – nominal 50,000 gallons/yr.
- Demonstration Plant – nominal 1.4 million gallons/yr.
- Commercial Plant – 20 to 30 million gallons/yr.
- Japanese Partners building commercial plant – complete this fall

Celunol Pilot Plant

- 50,000 gpy capacity
- Semi-continuous operation
- Separate hemicellulose and SSF fermentations with option for SSCF
- Enzyme production using in-licensed technology
- Beer still to produce 100 proof product.

Celunol Demonstration Plant

- Celunol is moving forward with plans to build a 1.4 million GPY demonstration plant in Jennings, LA.
  - Permits have been filed
  - Ground breaking September/October 06
  - Mechanical completion scheduled for 2nd Quarter 07
  - Purchasing long-lead time equipment
  - Will process multiple feedstocks including bagasse, dedicated energy crops, and wood
  - Will plant 10 to 20 acres of energy cane this fall

Commercial Project Development

- Celunol is developing a pipeline of site-specific 25 million GPY cellulosic ethanol projects using the following feedstocks:
  - Project 1: bagasse and energy cane
  - Project 2: waste wood and dedicated energy crops
  - Project 3: wood