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NIST/DOE Energy-Related Invention Project Final Report
Project DE-FG01-94CE 15594
4/1/94 through 2/28/97

**A Low-energy Continuous Reactor-Separator for
Ethanol from Starch, Whey Permeate,
Permeate Mother Liquor, Molasses or Cellulosics**

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Project Co-sponsors:

Permeate Refining Inc./ pilot project site host
Great Lakes Governor's Biomass Council
Bio-Process Innovation, Inc
Lamont Sign Co. (Stainless Steel Fabricators)
DOE Industrial Waste Program
Xylan/Xymax
Indiana Biomass Energy Program
Purdue University

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Abstract

In this project, a novel bio-reactor technology in which reaction is coupled with product separation was developed to pilot / demonstration scale. Combining reaction with separation during a fermentation allows the fermentation of highly concentrated feeds and allows the fermentation of streams with high levels of salts/non-fermentable inhibitors. Simultaneous saccharification and fermentation of polysaccharides such as starch and cellulose can also be combined with ethanol separation in the Continuous Stirred Reactor Separator (CSRS). Application of the bio-reactor to various substrates was investigated on a lab scale with fermentation of raw starch, cane molasses, xylose, whey permeate and permeate mother liquor. Flocculating yeast strains for high density sucrose/glucose fermentation were selected and adapted to form fast settling pellets. A strain of *K. marxianus* capable of fermenting high salt permeate mother liquor was also selected and adapted.

A low energy solvent ethanol recovery system was developed for ethanol recovery from the vapors leaving the reactor/separator. This Solvent Absorption/ Extractive Distillation (SAED) process gives a low energy method for purifying the ethanol to an anhydrous product. The amount of energy needed to recover an anhydrous ethanol product from a CSRS stage running at 8% ethanol was calculated to be under 8,000 BTU/ gallon. This process may also have further application in VOC (volatile organic carbon compounds) removal from air streams.

During this project, a 24,000 Liter CSRS was designed, fabricated, installed, and operated at a small batch ethanol plant (Permeate Refining Inc) in NE Iowa. The reactor was operated on a semi-continuous basis over a period of 18 months. A Solvent Absorption Extractive Distillation (SAED) system was also recently completed and installed at the Permeate Refining Inc. site for ethanol recovery/dehydration.

Commercialization of the technology is being actively pursued with negotiations for installation of the technology in a Chicago area plant currently underway, and possibilities for using the yeast strains on permeate mother liquor in Minnesota and Wisconsin are also being explored.

Table of Contents

	Page
Abstract	2
Introduction and Project Rationale.....	4
Project Objectives and Accomplishments.....	7
A. Develoment of a Flocculent yeast strain for Sucrose/ Glucose/ Fructose fermentations.....	7
B. Development of a Osmoltolerant yeast strain for Whey Permeate/ Permeate Mother Liquor fermentations.....	8
C. Lab scale (4 L) demonstration of the CSRS for raw starch-.....	14
D.Development of pretreatment techniques for biomass streams.....	16
E. Bench scale development of a flocculent yeast strain for xylose fermentation.....	18
F. Application of the CSRS to biomass hydrolysate in a Dual Culture Multi- Stage CSRS.....	19
G. Design of a Solvent Absorption Extractive Distillation Process for Ethanol Recovery from Vapor Streams.....	19
H. Design, Construction, and Performance of a 24,000 L CSRS- SAED system.....	20
Conclusions and Suggestions for Further Research.....	21
Bibliography.....	22
 Appendix A. Osmotic Inhibition of Free and Immob K.marx.....Dale et al (1994)	
App. B. High Density Ferm. w/ Flocc. Yeast.....Dale & Zhao (research rpt)	
App. C. Conv. of Raw Starch to Ethanol..... Dale & Lei (research rpt)	
App. D. The XDP for Biomass Conv. to Eth.....Dale et al. (1995)	
App. E. Xylose & Xylose/Glucose Ferm. by Yeast.....Zhao & Dale (research rpt)	
App. F. A Pilot CSRS for Ethanol from Biomass....cond. ERIP follow on propos.	
App. G. Design of Pilot CSRS w/ SAED.....Dale, Moelhman and Zhao (1995)	
App. H. Performance of a 24,000 L CSRS.....Dale, Gibb, and Lehman (1996)	

List of Figures

Figure 1. Conceptual use of CSRS on Raw Starch.....	6a
Figure 2. Energy Use to Recover Anhydrous Ethanol from Fermentation Broths: CSRS/SAED vs. Conv. Distillation.....	6b
Figure A.1 Adapted highly Flocculent Fermentation Yeast for CSRS type Reactors...BPY- 11.....	8a
Figure B.1 Purified Lactose Production from Whey.....	8b
Figure B.2 Osmolality of Salt, Lactose, Ethanol.....	8c
Figure B.3 Specific growth rate of <i>K. marxianus</i> 665 as a function of solution osmolality (ethanol and NaCl used to adjust broth osmolality).....	9a
Figure B.4 Specific growth rate of <i>K. marxianus</i> BPKY-15 as a function of solution osmolality (ethanol and NaCl used to adjust broth osmolality).....	9a
Figure B.5 Fermentation of lactose with different initial concentrations of ethanol using BPKY-15, brix versus time.....	9b
Figure B.6 Fermentation of lactose with different initial concentrations of ethanol using BPKY-15, OD versus time.....	9b
Figure B.7 Fermentation of lactose with different initial concentrations of salt using BPKY-15, Brix versus time.....	9c
Figure B.8 Fermentation of lactose with different initial concentrations of salt using BPKY-15, OD versus time.....	9c
Figure B9. Specific productivity of K.m. 665 as a function of osmolality as adjusted with salt and ethanol.....	9d
Figure B10. Specific productivity of K.m. BPKY-15 as a function of osmolality as adjusted with salt and ethanol.....	9d
Figure B. 11. Fermentation of PML of different initial brix (lactose/salt concentrations) using BPKY-15.....	10a

Figure B 12. Estimated Broth Osmolality in 15 brix PML (150 g/L lactose, 38 g/L salts) as a function of reaction completion.....	10b
Figure B 13. Estimated Broth Osmolality in 17 brix PML (170 g/L lactose, 42 g/L salts) as a function of fermentation reaction completion.....	10c
Figure B 14. Estimated Broth Osmolality in 19 brix PML (190 g/L lactose, 47 g/L salts) as a function of fermentation reaction completion.....	10d
Figure B. 15 Predicted Ethanol, Lactose, and Cell Conc. of a Three Stage Cascade CSTR with 15 brix Feed.....	11a
Figure B. 16. Predicted Outlet Ethanol Concentration from a 3 stage Cascade Fermenter as a function of residence time per stage with PML feed.....	11
Figure G.1. The SAED concept for ethanol recovery from a gas phase.....	19a
Figure G2. Schematic of SAED for PRI site.....	19b
Figure H.1. A Design Schematic of 24,000 Liter CSRS.....	20a
Figure H.2. A Photograph of the 24,000 L CSRS at the PRI site (vertical-looking down).....	20b
Figure H.3 The Design of a CSRS Stage.....	20c
Figure H. 4 A Photograph of a CSRS Stage.....	20d
Figure H. 5. A Photograph of a CSRS sieve tray separation stage.....	20f
Figure H. 6 A Design Schematic of the the SAED system at the PRI site....	20g
Figure H. 8. A Photograph of the installed SAED system at the PRI site.....	20h
Figure H. 9 Performance of the CSRS as a Cascade Reactor for 9/95 through 1/96.....	20i
Figure H. 10 Performance of the CSRS as a Cascade Reactor for 2/96 through 6/96.....	20j

List of Tables

Table 1 Conventional Corn Ethanol versus the CSRS Process.....	5a
Table 2. The CSRS versus Batch or Cascade CSTR fermentations.....	7a
Table B.3 Model Predictions of CSRS Performance of the CSRS on PML	14a
Table B.4 Model Preditions of CSRS Performance of the CSRS on PML with 2 g/L cell recycle to Stage 1.....	14b

Introduction and Project Rationale

Current batch fermentation technology for ethanol production requires large scale operations (12-50 million gal/yr of ethanol), a large capital investment (\$2-4.00/ annual gallon), and is energy intensive with an energy usage of 85-150,000 BTU/gal of ethanol produced from corn. About half of this energy is associated with drying and evaporating the stillage, and half with fermentation and distillation. Current ethanol production level in the USA is at about 800 million gallons/yr. The total market for ethanol as a 10% blend in gasoline would be 12 billion gallons. As more ethanol is produced, it is important to the USA's net energy position that ethanol be produced domestically in an energy efficient fashion. A Continuous Stirred Reactor Separation (CSRS) was developed over the course of this project. The CSRS is a new type reactor which allows simultaneous saccharification, fermentation, and ethanol separation in a combined process. Combining these reactions allows significant improvements in each operation. The CSRS offers a low energy, reduced capital, and reduced labor process for the production of ethanol from starch, molasses or cellulose. The CSRS system allows both high concentrations and low levels of substrate to be converted ethanol with low net energy consumption for ethanol recovery. Energy requirements for corn or grain to anhydrous fuel ethanol have been estimated at only 8,500 BTU/gal for smaller scale operations where the distillers grain is fed wet, and 28,500 BTU/gal for larger operations where the spent grain is dried. This represents a reduction of 70 to 91% of energy production cost for each gallon of ethanol produced. Total savings to the USA as this technology is implemented and retrofitted could be as high as 8×10^{13} BTU/yr. As our nations oil supplies are depleted, and clean air requirements are stiffened, the need for ethanol fuels is becoming a national priority. If, however, current high energy technology (utilizing coal fired boilers) uses 90 to 150,000 BTU's of coal energy to produce 84,000 BTU's of liquid ethanol fuel energy per gallon, the net effect is to produce a lot of coal fired boiler stack gas emissions to reduce car exhaust emissions, with the net effect on the US environment being open to debate. We thus feel there is a strong need for this new low energy ethanol production technology in the USA.

The basic schematic and concentration profiles in the CSRS are shown in Figure 1 for a raw starch fermentation. Stirred tank type reactors are operated in series, with the liquid streams moving from tank to tank contacted with a stripping gas to remove the ethanol product. Saccharification is sped by the reduction of sugar concentration as the sugar is fermented to ethanol. Fermentation is sped by the removal of the toxic ethanol product, and ethanol purification and concentration costs are reduced by the enrichment of the ethanol in the vapor phase. The gas stream is co-current to the tank to tank liquid flow in the enriching section, and counter-current in the stripping section. The final effluent from the CSRS is ideally characterized by complete saccharification of all polysaccharides, complete fermentation of sugars to ethanol and complete removal or separation of the ethanol into the gas phase.

High concentrations of sugars are associated with dry starch or grain substrates, concentrated whey permeate, or molasses streams. The advantages of fermenting highly concentrated feeds is a reduced volume of reactor effluent. This translates into savings in capital and energy used for concentrating and drying the residual fermentation solids (distillers dry grain from corn-ethanol operations). The CSRS can be configured so as to produce only a wet solids stream (wet distiller's grain) for a corn-ethanol process as shown in Figure 1. Initially dry starch feeds such as grain, or highly concentrated sugar streams such as molasses can be successfully fermented in the CSRS with only minimal dilution. Incorporating separation with fermentation eliminates the normal coupling between inlet sugar and final ethanol concentrations (0.51 g eth/g sugar theoretical conversion). This coupling usually limits feeds to about 20% fermentable solids due to ethanol toxicity at 8 to 12% ethanol (2.4-2.8 Os/kg solution osmolality). With the CSRS, this limit is removed because ethanol is removed from the fermenter as it is formed. The limit on feed concentration becomes the ability of the fermenting organism to tolerate highly concentrated feeds.

Organisms can be chosen which are more tolerant of high osmotic environments (or adapted to high osmotic as performed as part of this project). Dale et al (1990), showed the long term ability of a strain of *S. pombe* to ferment 50% solids molasses or up to 60% sucrose solutions in an immobilized cell reactor separator. In an immobilized reactor, conditions at each point in the reactor must allow some growth for stable reactor performance (Dale et al 1990b), while for the CSRS, as long as there are conditions for growth in some of the reactors, stable performance can be maintained (as long as net cell growth rates exceed loss and death rates). The CSRS can be designed to handle starch streams of 35 to 45% T.S. and molasses streams of up to 45% T.S., or concentrations about two times higher than normal. The limits of total solids which can be handled by the system are set either by the ability of the fermenting microbes to withstand highly concentrated environments or the total viscosity of the fermentation broth.

Table 1 shows estimated energy use for a conventional corn ethanol process versus the CSRS ethanol process (utilizing solvent absorption of the ethanol vapors from the CSRS). Total energy requirements for the conventional system are determined at about 23,000 BTU/gal of ethanol produced, while the CSRS system has a energy use of only 8,500 BTU/gal. if the spent grain is fed wet, and 28,500 BTU/gal if the spent grains are dried.

Low concentrations of sugars are associated with cheese whey, cellulose hydrolysate, papermill 'sulfite liquor', or waste liquid starch streams exiting from a corn or potato processing plant. These streams usually have to be concentrated prior to fermentation in order to reduce ethanol distillation costs. Distillation energy requirements grow rapidly when feed ethanol concentrations are under 5% ethanol (less than 10% initial fermentable solids) as shown in Figure 2. Figure 2 also shows the estimated energy usage for the CSRS as a function of maximum ethanol concentration (first stage of stripper section). Feed

streams producing as little as 3% maximum ethanol can give a 70% ethanol product using the CSRS with solvent absorption for under 7,000 BTU per gallon (anhydrous basis). Energy savings over conventional systems are apparent in Figure 2. A 3% ethanol feed taken to a conventional distillation column would require a distillation energy of 32,000 BTU/gal versus 6,500 BTU/gal with the CSRS, an 80% reduction in energy use.

Detailed Description of Invention

The CSRS concept is shown in Figure 1. The concept incorporates conventional stirred tank in series reactor technology with co- and counter-current gas phase product stripping from the fermentation broth as the broth moves from stage to stage. This separation has the effect of decreasing the ethanol concentration in the stirred tank reactors, thus increasing the rate of ethanol production by the cells. The cell density is maintained in the reactor system either by continuous cell growth, by immobilizing the cells on beads or other matrix, through use of flocculent strains of organisms, or by recycling the cells from the effluent stream to the inlet stream. In this project, we have focused on the use of self aggregating or flocculent yeasts. The rate of ethanol production can be determined as a function of the environment in each stirred reactor.

Ethanol production by yeasts or ethanolic bacteria (*Z. mobilis*) is inhibited both sugars (substrate) and product (ethanol). Salts and other by-products can also be inhibitory. We recently surmized that inhibition of these organisms by substrate, product, and salts could all be related to a single phenomena, solution osmolality or water activity. A recent paper by Dale et al (1997, Appendix A) showed that this model for yeast growth and productivity accurately predicted inhibition by both substrate (sugars) and product (ethanol). This model is also very useful in predicting the combined effects of salts or other osmotically important components of a fermentation broth on fermentation rates.

We proposed that growth and inhibition are simple linear functions of osmolality for most fermentation yeasts and bacteria. Growth is slightly more inhibited than fermentation, with growth completely inhibited at osmolality of over 2.3 ($\kappa_{\epsilon\mu m} = 2.3$) for standard strains of *S. cerevisiae*. Productivity for most *S. cerevisiae* ceases at an osmolality around 3.0 ($\kappa_{\epsilon vm} = 3.0$). Thus specific productivity and growth can be written as simple functions of solution OS.

$$v = v_m [1 - \epsilon / \kappa_{\epsilon vm}] \quad \text{Eq. 1}$$

$$\mu = \mu_m [1 - \epsilon / \kappa_{\epsilon \mu m}] \quad \text{Eq. 2}$$

The osmolality of the solution can be determined as a simple additive function of the osmolality of the various components of the solution broth.

Table 1. Energy use in CSRS No-Cook versus Conventional Batch Processing of Corn (BTU/ gallon of ethanol)

	<u>Conventional</u>	<u>CSRS</u>
1) Cook	15,000	—
2) Fermentation cooling	-3,500	-3,500
3) Distill to 180 proof (dist. cooling)	15,000 -16,000	5,000 -6,000
4) Dehydrate (cooling)	15,000 (iso-propyl) -16,000	3,500 -4,000
5) Evap thin stillage	18,000	—
6) Dry distillers grain	20,000	20,000
TOTAL HEAT	83,000	28,500
TOTAL COOLING	-35,500	-10,000

Table 2

Comparison of Ethanol Fermentation Processes
(2 Million Gal/Yr Scale)

Reactor Type	Reaction Rate (g/l*hr)	Res. Time (hr)	React. Vol. (gal)	Capital Cost for fermenter
1) Batch	2.0	48	[24,000]*7	\$1,066,000
2) CSTR (1 stage)	0.6	160	300,000	\$545,000
3) CSTR (3 stage)	3.7	24	[16,000]*3	\$372,000
4) CSTR (5 stage)	4.9	15	[7,500]*5	\$425,000
5) CSRS (5 stage)	35.5	2.5	[1,000]*5	\$155,000
6) CSRS (8 stage)	41.3	2.2	[600]*8	\$192,000

6b

Figure 1 The CRS concept with simultaneous starch hydrolysis in a 'no-cook' process.

6c

Figure 2. Energy Use to Recover Anhydrous Ethanol from Fermentation Broths:
CSRS/SAED vs. Conv. Distillation

$$\varepsilon = \varepsilon_{\text{sug}} + \varepsilon_{\text{eth}} + \varepsilon_{\text{inhib}}$$

Eq. 3

The advantages of the CSRS technology over batch and conventional stirred tank technology are described in Table 2. By removing ethanol from the fermentation broth, solution osmolality is decreased and net fermentation rates are improved. For a basal case of 20% glucose feed, 50 g/l cells in the fermentation broth, the fermentation performance of several fermenters were calculated. Using a corn to ethanol plant at the 2 million gallon ethanol/yr scale, the fermenter size and costs were calculated. For batch operations, seven 24,000 gallons fermenters would be required, while for a single stage CSTR (Continuous Stirred Tank Reactor) with final ethanol concentration of 98 g/l, a 123,000 gallon tank would be required. Fermentation performance is improved when three CSTR's are run in series. Average reactor productivity jumps from 0.6 g/l-hr to 3.7 g/l-hr as only the final stage of the reactor is seeing the 98 g/l-hr ethanol (OS= 2.4 Os/kg) which is quite inhibitory. Average productivities rise again when five stages are utilized, but net capital cost is higher due the added expense of more fermenters. The CSRS has a productivity of 35.5 g/l-hr with complete fermentation of a 20% feed in a 2.5 hour residence time. Ethanol levels are held to under 50 g/l on any stage to maximize fermentation rates and minimize ethanol catalyzed cell death. Captital cost for the CSRS with 5 stages is only 15% of the cost of a batch reactor system. As with the CSTR's, added stages, while improving average productivity, cause net capital cost to be somewhat higher (an 8 stage CSRS costing more than a 5 stage system).

Project Objectives and Accomplishments

The basic objectives of this project were to demonstrate use of the CSRS technology on various substrates, develop the low energy solvent absorption/ extractive distillation (SAED) process and to build and demonstrate the CSRS/SAED technology on a pilot or small commercial scale. These objectives were met as listed below:

A. Develoment of a Flocculent yeast strain for Sucrose/ Glucose/ Fructose fermentations-

A high cell density in a stirred reactor can be maintained by 1) recycling the cells or by retaining the cells in the reactor. Cells can be retained in one of three basic ways, 1) self-immobilization through the formation of floccs, which settle at a high rate as compared to free yeast, immobilization of the microbes on a support material which is maintained on the stage. This support medium could be gel beads or small fibrous packing units. In this project, we have focussed on flocculent yeast as being the simplest and cheapest means of maintaining a high cell density in the reactor. Our strain selection and selection methods for finding a top performing flocculent yeast strain are decribed in Appendix B.

We developed a strain of flocculent yeast which has the ability to form clumps ranging from 0.25 mm to 3-5 mm ID. These clumps tend to settle rapidly, with a settling time of 15 to 30 seconds in a 1 liter flask. With this strain, very high cell densities can be maintained in the bioreactor. In repeated batch and stirred reactors, we reached 20-40 g/l, while in an upflow tower reactor, a density of 80 g/l was reached. To a large degree, fermentation rates are a linear function of cell density (if sugars and ethanol are constant). In batch fermentations with standard non-flocculent yeast, an initial yeast density of 0.5 g/l reaches 15 g/l by the end of the fermentation. If a live cell density of 40 g/l can be maintained in the CSRS, we calculate complete conversion of a 30% sugar feed in a residence time of under 12 hours.

We feel that this yeast, BPY-11, may have excellent commercialization potential for the ethanol industry. A photo of these yeast is shown in Figure A.1. Selection of this strain is described in Appendix B, and the strain was adapted to remain in this flocculent stage through the operation of a small (1 liter Bio-ferm) stirred reactor. The strain was then used in our 24,000 L. reactor as described in Appendix I, and was also utilized in a 225 KL (kiloliter) 5 stage cascade fermentation designed by Dale and implemented at PRI for their main fermentation system to replace their batch operations.

B. Development of a Osmotolerant yeast strain for Whey Permeate/ Permeate Mother Liquor fermentations

Approximately 5-10% of whey produced in the USA is used for the production of purified lactose or about 250 million #/yr (Dale, 1996). A basic flow sheet for lactose production is shown in Figure B. 1. We see that the permeate feed to the process is 80% lactose, and approximately half of this is recovered as purified lactose. The remainder stays with the 'molasses' or permeate mother liquor (PML). The salt fraction in the permeate (about 10% dry basis) increases to 20% in the PML. The ability of yeast to ferment the lactose in PML was recently evaluated.

1) *Selection of Yeast*- the ability of 10 strains of highly productive ethanolic lactose fermenting *K. marxianus* type yeast to grow at a various salt concentrations was determined. Growth at 25, 35 g/L NaCl was tested with 50 g/l lactose. (Osmolality of lactose, glucose, salt, and ethanol as a function of concentration are shown in Figure B. 2. Osmolalities are simply additive at lower concentrations, i.e. the osmolality of a lactose, salt, and ethanol solution can be determined as the sum of the osmolalities of the components.) The four best performing strains, i.e. those showing higher growth rates at the higher salt concentrations, were tested at 35, 45, and 55 g/L concentrations and the cell densities measured as a function of time.

Figure B.1 Purified Lactose Production from Whey

Figure B.2 Osmolality of Salt, Lactose, Ethanol

2) *Adaptation of Yeast* The best performing yeast strain, BPKY-15, was then adapted to high salts by continuously growing and transferring the culture for a period of 2 months in a 35 g/L NaCl solution. Tests on the ability of this adapted strain to ferment broths with high salts was then tested. The strain, BPKY-15, was tested for growth and fermentation performance in various test salt/lactose solutions. Figure B. 3 and B. 4 show the inhibition of growth of *K. marxianus* NRRL 665 as compared to BPI's BPK-15 strain. It can be seen that salt seems slightly more inhibitory than ethanol for both strains, although only slightly. The 665 strain showed near complete inhibition with salt at an osmolality of 1.5 os/kg, while the BPKY-15 strain showed only an 85% inhibition. Extrapolating the inhibition curves indicates a complete inhibition of 665 at 1.55, while the BPKY-15 strain will be completely inhibited at 1.75 os/kg, an improvement of about 15%. Both strains seem to show a complete inhibition of growth with ethanol at 1.75 os/kg (based on extrapolating the lines seen in Figure B.3 and B.4), an ethanol concentration of 75 g/l. Growth was measured by Optical Density (OD) at 660 nm. Growth is generally more inhibited than is productivity (ethanol production per gram yeast) by solution osmolality.

Lactose (Brix) and cell density as a function of time for fermentations with BPKY-15 are shown in Figures B.5 and B.6 as inhibited by increasing initial concentrations of ethanol(figure B. 5 and B. 6), and salt (Figure B. 7 & 8). The specific productivity, v , as defined by

$$v = (1/x) \, dP/dt \quad \text{Eq 4}$$

where x is cell density (g/L) and P is ethanol concentration (g/L) was determined from these charts, and a similar set of fermentations run with *K. marxianus* 665. Specific productivity (Eq. 4) determined from these experiments are shown in Figure B. 9 and B.10. In both of these figures we see that productivity is more strongly inhibited by ethanol than by salt (while growth showed similar inhibition for both salt and ethanol, and if anything was slightly more inhibited by salt). Parallel inhibition patterns were noted with each strain as indicated by the dotted lines seen in Figures B 9-10 with the fermentation being inhibited by approximately 0.7-0.75 os/kg more by the ethanol than by salt. The adapted BPKY-15 yeast showed a much better tolerance to the salt than does *K.m.* 665. A difference of 0.45 os/kg is noted, a 22% improvement in salt tolerance. Ethanol tolerance in the BPKY-15 is also better, with a difference of 0.3 os/kg. The BPKY-15 strain shows complete productivity inhibition at 1.75 os/kg (75 g/L ethanol) while the 665 strain shows complete inhibition at 1.5 os/kg (60 g/L ethanol). Both strains seem to show growth and productivity patterns being identically inhibited by ethanol osmolality, i.e., the phenomena of productivity being less inhibited than growth is not evident in this set of data.

From the data sets obtained, the adapted BPKY-15 yeast can be seen to be clearly superior to another highly productive lactose fermenting *K. marxianus* strain, 665. Salt tolerance is improved by over 20%, and ethanol tolerance seems to also be about 20% higher. Growth showed similar inhibition rates

9a

K.m. 665 Growth

Figure B.3 Specific growth rate of *K. marxianus* 665 as a function of solution osmolality (ethanol and NaCl used to adjust broth osmolality)

BPKY-15 Growth

Figure B.4 Specific growth rate of *K. marxianus* BPKY-15 as a function of solution osmolality (ethanol and NaCl used to adjust broth osmolality)

9b

Figure B.5 Fermentation of lactose with different initial concentrations of ethanol using BPKY-15, brix versus time.

B5

Figure B.6 Fermentation of lactose with different initial concentrations of ethanol using BPKY-15, OD versus time.

B6

9C
Figure B.7 Fermentation of lactose with different initial concentrations of salt using BPKY-15, Brix versus time.

Figure B.8 Fermentation of lactose with different initial concentrations of salt using BPKY-15, OD versus time.

9d
Figure B9. Specific productivity of K.m. 665 as a function of osmolality as adjusted with salt and ethanol.

Figure B10. Specific productivity of K.m. BPKY-15 as a function of osmolality as adjusted with salt and ethanol.

between salt and ethanol osmolalities, but productivities were clearly less inhibited by salt osmolality than by ethanol, although the slopes were parallel.

3) *Effect of Osmolality on Fermentability of PML* - We have suggested that osmolality can be used as a simple model for growth and productivity of ethanolic yeasts and bacteria (Dale et al, 1994). PML offers a good model for this system. The osmolality of the salts in whey permeate can be determined by difference from osmolality data presented by Dale et al. (1994). Fermentation rates of BPKY-15 in PML at various concentrations are shown in Figure B. 11. Cell growth and brix drop decreased at the higher brix feeds. When 21 brix PML was fermented with K.m. 665, no activity was noted (data not shown), another indication of the superiority of the BPKY strain. Complete utilization of lactose was noted in the 15, 17, and 19 brix fermentations, but lactose remained in the 21 brix fermentation after 92 hours of fermentation. Total solution osmolality was estimated as a function of fermentation completion for PML as shown in Figures B. 12, 13, and 14. As per these graphs, total solution osmolality increases as the lactose is converted to ethanol. For 15 brix PML (150 g/l initial lactose, 38 g/l salts) the initial osmolality increases from 1.4 os/Kg to 2.5 at reaction completion (7% ethanol). Similarly, 17 brix PML has an initial estimated osmolality of 1.6 increasing to 2.8 os/Kg at reaction completion of 85 g/L ethanol, and 19 brix PML has an initial osmolality of 1.8 increasing to 3.2 os/Kg.

4) *Fermentation of PML in a Cascade Reactor- Design Performance-* A computer modeling program was written to predict the performance of a multi-stage ethanol continuous cascade reactor. The effect of residence time, feed strength, and number of reactors in series was determined. A model for performance of the cascade reactor system was constructed based on either 1) a immobilized cell density in the reactors (as with flocculant cells or cells held on beads, etc), 2) free cells , and 3) free cells with cell recycle using the Dale osmolality model for growth and productivity (Equations 1 and 2). The basic performance of a continuous reactor can be modeled by a basic mass balance on each of the components, cells, sugar, and ethanol. Each of these components affect the rate of cell growth and consequent cell concentration in the reactor stage. Kinetics of continuous stirred tank microbial reactors (CSR) have been studied extensively over the years. The basic analysis for a single stage reactor (with no input cells) at steady state simply indicates that growth rates must equal the dilution rate.

$$D = \mu$$

Eq. 5

If, as we are proposing, μ is a function of osmolality, then a certain dilution rate will correspond to a certain solution osmolality at which cell growth will equal dilution rate.

Figure B. 11. Fermentation of PML of different initial brix (lactose/salt concentrations) using BPKY-15.

B11

106
Figure B 12. Estimated Broth Osmolality in 15 brix PML (150 g/L lactose, 38 g/L salts) as a function of fermentation reaction completion.

100
Figure B 13. Estimated Broth Osmolality in 17 brix PML (170 g/L lactose, 42 g/L salts) as a function of fermentation reaction completion.

Figure B 14. Estimated Broth Osmolality in 19 brix PML (190 g/L lactose, 47 g/L salts) as a function of fermentation reaction completion.

B14

10e

21 Brix PML

A model of a 3 stage cascade reactor system was written in BASIC. The mass balances on the stages were written as:

$$X_{ss(n)} = X_{ss(n-1)} + X_{ss(n)} \cdot \tau \cdot \mu \quad \text{Eq. 6}$$

$$P_{ss(n)} = P_{ss(n-1)} + X_{ss(n)} \cdot \tau \cdot v \quad \text{Eq. 7}$$

$$S_{ss(n)} = S_{ss(n-1)} - \Delta P_{(n)} / Y_{p/s} \quad \text{Eq. 8}$$

where $X_{ss(n)}$ is the steady state concentration of cells (g/L) on stage n , and $P_{ss(n)}$ & $S_{ss(n)}$ are the concentrations of ethanol and sugar on stage n , and τ is the residence time on the stage (Volume of reactor divided by the feed rate per hour). μ and v as were written as functions of osmolalites, dependent on the sum of osmolality of salt, ethanol and sugar on the stage as per Equations 2 and 3.

This system of equations can be solved iteratively to find steady state concentrations of cells, ethanol and sugar. Basically, the model predicts a steady state cell density and ethanol concentration and net cell productivity as a function of final osmolality on the stage. The cell density and outlet ethanol increase as the residence time per stage increases. The predicted ethanol, lactose, cell, and osmolality concentration on each of the stages of a 3 stage continuous cascade reactor is shown in Figure B. 15 for a residence time of 15 hours per stage. The ability of the system to produce higher ethanol as residence time (and feed concentration) are increases is shown in Figure B. 16. We see that there is an asymptotic approach to approximately 85 g/l outlet ethanol, which is the limit imposed by salts plus the ethanol.

Figure B. 16. Final Ethanol Conc. in a 3 Stage PML Cascade Reactor as a Function of RTD per stage.

11a

15 Brix PML

Figure B. 15 Predicted Ethanol, Lactose, and Cell Concentrations of a Three Stage Cascade CSTR with 15 brix Feed.

Steady state performance of a 3 stage cascade reactor was predicted for three feeds, 1) PML (20% salts, 60% lactose d.b.), 2) Whey Permeate Concentrate (10% salts, 80% lactose d.b.) and 3) purified lactose. Inhibition constants of $K_{\text{sum}} = 2.3$, and $K_{\text{gvm}} = 2.9$ were used to model yeast growth and productivity. Steady state sugar (lactose) concentrations, ethanol concentrations, cell densities, osmolality and productivities of the 3 stage reactor system running at a constant residence time, t , of 15 hours per stage (45 hours total) are shown in Table B. 1

Table B.1 . Predicted Performance of a Three Stage Cascade Reactor for Lactose					
Lactose	Ethanol	Cell Dens	Osmol.	Productivity	
PML- 25% Salts					
160.00			1.27		
70.58	41.21	4.03	1.80	2.75	
18.01	65.42	4.82	2.22	1.61	
3.04	72.36	5.00	2.35	0.46	
WPC- 12% Salts					
200.00			1.21		
98.80	46.63	4.35	1.81	3.11	
37.40	74.92	4.44	2.29	1.89	
10.09	87.51	4.61	2.52	0.84	
Lactose- 0% Salts					
240.00			1.07		
118.57	55.96	5.08	1.80	3.73	
47.60	88.67	5.28	2.35	2.18	
13.96	104.13	5.48	2.64	1.03	

As shown in Table B. 1, the osmolality reached in stage 1 is identical for each substrate, 1.80 Os/kg. osmolality reached on subsequent stages is affected by the inlet cell density and thus vary slightly depending on the substrate. However, the inhibitory effect of the salts is easily noted. An ethanol concentration of 104 g/L is reached with no salts, an ethanol concentration of 87 g/L is reached using WPC with a 12% salt make-up, while only 72 g/L ethanol is obtained from the salt enriched PML. Reactor productivities drop also as seen in the last column, the PML average productivity over the three stages, 1.61 g/L-hr, is 30% lower than for a pure lactose feed.

The effect of cell recycle is shown in Table B. 2 with the same basic conditions (15 hr RTD/ Stage and same yeast kinetics). We can see that with recycle yeast, outlet ethanol concentrations are raised by about 8 to 10 g/l, and productivities are boosted about 10%, with most of the gain noted in the Stage 1.

	Table	B.2 Predicted Performance of a Cascade Reactor w/ Cell Recycle			
	Lactose	Ethanol	Cell Dens.	Osmol.	Productiv
	PML- 25% Salts				
Feed	180		3	1.43922	
Stage 1	76.6614	47.6215	5.505587	2.06688	3.17477
Stage 2	22.9342	72.3836	5.72009	2.49726	1.65081
Stage 3	4.9548	80.6766	5.942951	2.64959	0.55287
	WPC- 12% Salts				
Feed	220		3	1.30659	
Stage 1	98.9953	55.7626	6.084933	2.03977	3.7175
Stage 2	35.0061	85.2535	6.322008	2.54447	1.96607
Stage 3	9.19672	97.1734	6.568321	2.77337	0.79466
	Lactose- 0% Salts				
Feed	250		3	1.1275	
Stage 1	106.421	66.1653	6.991678	2.00856	4.41102
Stage 2	35.4932	98.8453	7.264082	2.57754	2.17867
Stage 3	8.7664	111.192	7.547098	2.81944	0.8231

With the addition of cell recycle, the dilution rate does not set growth rates, and differing stageosmolality are seen in stage 1 of the differing feeds.

An industrial scale fermentation of PML was initiated in late 1996 by Minnesota Clean Fuels based on design input from BPI. Performance of this system was noted to fairly closely follow the design calculations shown here.

5) *Fermentation of PML in a CSRS- Design Performance.* The CSRS type reactor is excellent for feeds such as PML, with its high salts. By removing ethanol from the fermentation broth, stageosmolality are lowered, allowing complete utilization of the lactose. A four stage CSRS system was modeled, with no ethanol stripping in the enricher. The system consists of two enriching stages and two stripping stages. Table B. 3 shows the performance of the

system without cell recycle, and Table B. 4 shows the predicted performance if 2 g/L cells are returned with the feed.

In Table B. 3. (no cell recycle) we see that the CSRS can be operated at an RTD per stage of under 10 hours for a 170 g/L lactose PML feed (25% salts in the feed). RTD increases to 11 hours for a 180 g/L lactose feed, and eventually to 18 hours/stage at a 200 g/L feed. Higher feeds than this are not practicable due to the inhibition of cell growth due to the salts.

However, if cells are recycled, higher levels of feed and shorter RTD's can be attained as shown in Table B.4. These are shown graphically in Figure B. 17

Figure B. 17. Feed Lactose Strength in CSRS as a function of RTD per stage with and without cell recycle.

Even with cell recycle, a feed strength of 210 g/L lactose is the maximal which can be fermented in the CSRS, again because of the inhibition of salts at this concentration of feed. At 210 g/L feed, the salts in the PML alone are contributing an osmolality of about 0.9 Os/kg.

C. Lab Scale Demonstration of the CSRS for Raw Starch- Simultaneous Saccharification and Fermentation and Separation

The simultaneous saccharification and fermentation (SSF) of starch or cellulose allows improved saccharification kinetics due to removing glucose as it is formed. The reduction of glucose concentration has two desirable effects on the kinetics: 1) reducing product (glucose) inhibition of the forward reaction, and

Table B.3 Model Predictions of CSRS Performance of the CSRS on PML

	Lactose	Ethanol	Cells	μ	v	osm.	
Feed	170.0		0.1				tau=9
Enr #1	152.0	8.6	1.0	0.109	1.0	1.5	
Enr #2	106.7	30.1	3.2	0.074	2.4	1.7	
Str #3	70.0	47.2	3.7	0.016	1.9	2.2	
Str #4	23.7	53.1	5.7	0.037	2.4	2.0	
Feed	170.0		0.1				tau=10
Enr #1	128.5	19.7	2.3	0.094	2.0	1.6	
Enr #2	74.4	45.5	4.2	0.044	2.6	2.0	
Str #3	16.4	60.4	7.1	0.040	2.8	2.0	
Str #4	1.0	43.8	13.4	0.044	1.2	2.0	
Feed	180.0		0.1				tau=12
Enr #1	132.2	22.8	2.4	0.077	1.9	1.7	
Enr #2	79.5	47.9	3.7	0.028	2.1	2.1	
Str #3	20.7	62.3	5.9	0.033	2.3	2.0	
Str #4	1.0	44.3	11.6	0.039	0.9	2.0	
Feed	180.0	tau	0.1				tau=11
Enr #1	141.1	18.5	2.1	0.084	1.7	1.7	
Enr #2	90.0	42.9	3.6	0.039	2.2	2.0	
Str #3	35.4	59.5	5.0	0.027	2.4	2.1	
Str #4	4.3	48.6	8.9	0.038	1.3	2.0	
Feed	190.0		0.1				tau=14
Enr #1	141.7	23.0	2.2	0.065	1.6	1.8	
Enr #2	91.9	46.7	3.0	0.020	1.7	2.1	
Str #3	35.1	62.1	4.2	0.021	1.9	2.1	
Str #4	4.1	49.9	8.0	0.033	1.1	2.0	
Feed	190.0		0.1				tau=16
Enr #1	156.0	16.2	1.3	0.071	1.0	1.8	
Enr #2	103.4	41.2	2.6	0.031	1.6	2.1	
Str #3	49.2	60.5	3.4	0.015	1.6	2.2	
Str #4	6.4	56.4	6.7	0.032	1.3	2.1	
Feed	200.0		0.1				tau=18
Enr #1	150.8	23.4	1.8	0.053	1.3	1.9	
Enr #2	104.2	45.6	2.3	0.011	1.2	2.2	
Str #3	60.0	56.8	2.5	0.004	1.2	2.3	
Str #4	11.4	57.7	4.5	0.025	1.3	2.1	

Table B.4 Model Predictions of CSRS Performance of the CSRS on PML with 2 g/L cell recycle to Stage 1.

	Lactose	Ethanol	Cells	μ	v	osm.	
Feed	180.0		2.0				tau=8
Enr #1	129.1	24.3	4.0	0.068	3.0	1.8	
Enr #2	79.1	48.0	5.2	0.028	3.0	2.1	
Str #3	28.3	58.1	6.8	0.030	3.0	2.1	
Str #4	4.5	43.2	10.1	0.039	1.4	2.0	
Feed	190.0		2.0				tau=10
Enr #1	135.9	25.8	3.7	0.051	2.6	1.9	
Enr #2	86.9	49.1	4.4	0.015	2.3	2.2	
Str #3	34.3	60.1	5.5	0.022	2.5	2.1	
Str #4	4.9	47.6	8.4	0.033	1.4	2.0	
Feed	200.0		2.0				tau=11
Enr #1	140.0	28.6	3.9	0.044	2.6	2.0	
Enr #2	94.7	50.2	4.0	0.002	2.0	2.3	
Str #3	47.2	58.8	4.5	0.010	2.1	2.2	
Str #4	9.4	52.1	6.2	0.026	1.6	2.1	
Feed	210.0		2.0				tau=13
Enr #1	150.0	28.6	3.5	0.033	2.2	2.1	
Enr #2	107.9	48.6	3.2	1.541	2.3		
Str #3	71.9	54.2	3.2	1.320	2.4		
Str #4	32.3	52.8	3.5	0.012	1.5	2.2	
Feed	210.0		2.0				tau=15
Enr #1	144.2	31.3	3.4	0.027	2.1	2.1	
Enr #2	102.3	51.3	2.9	0.001	1.3	2.4	
Str #3	62.4	56.3	2.8	0.001	1.267	2.3	
Str #4	20.1	54.2	3.7	0.016	1.3	2.2	
Feed	210.0		2.0				tau=17
Enr #1	149.5	28.8	2.9	0.020	1.7	2.1	
Enr #2	106.4	49.3	2.5	0.001	1.207	2.4	
Str #3	69.1	54.9	2.5	0.001	1.046	2.3	
Str #4	26.1	54.6	3.1	0.014	1.2	2.2	
Feed	210.0		2.0				tau=18
Enr #1	137.3	34.6	3.3	0.021	1.9	2.1	
Enr #2	96.5	54.1	2.5	0.001	1.081	2.4	
Str #3	52.3	58.5	2.6	0.003	1.2	2.3	
Str #4	10.9	53.9	4.1	0.020	1.1	2.1	
Feed	220.0		2.0				tau=20
Enr #1	152.4	32.2	2.8	0.015	1.6	2.2	
Enr #2	115.2	49.9	2.0	0.001	0.884	2.4	
Str #3	92.5	48.7	1.3	0.001	0.542	2.5	
Str #4	75.1	41.0	1.8	0.001	0.414	2.4	

2) reducing back polymerizations of glucose to undesired products. SSF of cellulose has been suggested and utilized by various researchers (Schell and Walter, 1991; Spindler et al, 1991; Bowman and Geiger, 1984). Enzyme kinetics for starch and cellulose saccharification are complex due to the number of multi-chain polymers of glucose which are generated during the breakdown of these polymers, as well as some reverse reactions. The kinetics of saccharification of dextrans by glucoamylase was recently modeled by Zanin and Demores (1995), and earlier by Kusunoki et al, (1982). Kinetics of raw starch hydrolysis were modeled by Matsumura et al (1988) who noted that glucose inhibition was more pronounced at lower temperatures (25 C) as was enzyme adsorption onto the raw starch, and reaction rates were slowed as well. High temperature (40 C) allowed much better performance of the glucoamylase on the raw starch.

The basic hydrolysis of raw starch involves 1) adsorption of the enzyme onto the starch granule, 2) a surface reaction where the enzyme releases a glucose monomer from the starch molecule, and 3) mass transfer of the glucose monomer from the starch granule into the bulk liquid phase. Matsumura et al (1988) further proposes that the enzyme has a specific site where glucose and the enzyme bind to form an inactive complex. Simultaneous fermentation with saccharification reduces product inhibition as the product (glucose) is removed to ethanol and the formation of undesirable side-products generally associated with the saccharification process of both cellulose and starch. Product (glucose) is more inhibitory to enzymatic cellulose conversion than starch conversion. In general, dextrin (cooked starch liquified with alpha-amylase) is fairly readily converted to glucose by gluco-amylase particularly at high temperatures (50-60C). Even here though, there is practical limit of about 20% total solids if over 95% conversion is desired (Solvay, 1988).

Raw starch conversion to glucose at fermentation temperatures is a slower process. We experimented with koji, a mix of wet starch impregnated with different strains of *Aspergillus*. Raw starch saccharification enzymes have been suggested in the literature as being developed by strains of *Aspergillus niger* and *A. amorami* (Ueda & Koba, 1980, Hayashida et al, 1982, Park and Rivera, 1982 and others), and *Chalara paradoxa* (Mikuni et al, 1987).

Raw starch conversion was demonstrated as a part of this project. We investigated the best mix and concentration of commercial enzymes required to convert the starch granules to sugars, and then further operated a 4 Liter CSRS for a period of 35 days using a raw starch feed. A summary of our CSRS work is included as Appendix C.

We concluded that:

1) *The system will work at a fairly high efficiency, 80-92% of the starch is converted.*

2) *Hydrolysis of the starch is the rate limiting step.* We maintained a high concentration of yeasts on the stages, with the result that glucose was held at very low levels on every stage except 1, where approximately 20 g/l glucose was routinely noted.

3) *At least a 24 hour residence time on four stages is required to allow time for the enzymes to work (at the levels we found 'optimal')*

4) *Some small (3-5%) fraction of the starch seemed to be resistant to the low temperature hydrolysis.*

We would conclude from this work that raw starch fermentation is certainly practicable, especially for whole grain systems (dry mill ethanol) where non-utilized starch can be sold with the spent 'distillers dry grain' (DDG). If, however, the non-utilized starch is lost as per a wet mill operation, the process would need further enzyme optimization to move the system towards 98-100% conversion of the starch.

D. Development of pretreatment techniques for biomass streams

The possibility of using the CSRS for cellulosics fermentations seems to be a natural application for this reactor. With the CSRS, one can combine saccharification, fermentation, and ethanol separation simultaneously- in a similar manner to the fermentation of raw starch, as discussed in Section 2C.

BPI will be developing a cellulosics to ethanol process during a follow-on project also supported by the ERIP program. The proposal for this effort is included as Appendix D. Some of this work has already been started, and a second low temperature, low chemical steeping delignification process is being developed by BPI.

Biomass is a mix of three basic components, lignin, cellulose and hemicellulose. Lignin serves as a sort of 'glue' giving the biomass fibers its structural strength, while hemicellulose and cellulose polymers are the basic building blocks of the fibers. In order to break down the hemicellulose and cellulose to sugars, the basic structure of the biomass must be attacked. Once the structure of the biomass is disrupted, the hemicellulose and cellulose can be converted to sugars enzymatically. There are two basic approaches to biomass breakdown to sugars, 1) acid hydrolysis with a variety of low acid-high temperature or high acid-low temperature conditions being suitable to both breakdown the structure of the biomass and release free sugars; and 2) enzymatic hydrolysis after some sort of pretreatment which allows enzymatic attack of the polymers.

Acid hydrolysis is perhaps currently seen as the most technologically mature method of sugar release from biomass. Acid (sulfuric or hydrochloric) can serve both for disruption and hydrolysis of the cellulosic polymers. Strong acid allows complete breakdown of the components in the biomass to sugars, but also requires large volumes of concentrated sulfuric acid and can result in the production of furfural, an inhibitory byproduct (Goldstein and Easter, 1992; Ladisch and Swartzkopf, 1991). Dilute acid allows reduced acid concentrations, but requires higher temperatures, and again gives furfural. Both concentrated

and dilute acid processes for biomass conversion are being pilot tested by TVA and NREL researchers (Barrier et al, 1986, Torget et al, 1995). The major drawback to these acid processes are the cost of acid and the requirement to neutralize the acid after treatment. NREL researchers are evaluating low acid high temperature-high pressure treatments to reduce the costs for acid, but these systems will require expensive reactors and are basically batch processes.

Enzymatic hydrolysis eliminates the need for large quantities of acid (and the neutralization of this acid) but enzyme costs are currently high, and enzyme attack of the cellulose and hemicellulose polymers can be slow. However, if fermentation occurs simultaneously with saccharification, enzyme rates can be maximized by reducing product inhibition. This simultaneous saccharification and fermentation (SSF) is being promoted by the NREL as perhaps the best process for enzymatic conversion of cellulose to ethanol (Schell and Walter, 1991, Spindler et al, 1991)

Ammonia explosion is a process being promoted by AFEX (B. Dale et al, 1985; AARC Bulletin, 1994)) which uses a quick pressure reduction after soaking the biomass with liquid ammonia solution. This system does not directly liberate any sugars, but allows the polymers (hemicellulose and cellulose) to be attacked enzymatically and reduced to sugars. Steam explosion and peroxide extrusion are similar processes. Steam explosion is being developed by Stake Technology which involves extrusion of the biomass at a high temperature and pressure, while peroxide extrusion uses a chemical pretreatment along with extrusion to accomplish the same goal of breaking down the internal structure of the biomass fibers. Steam explosion and peroxide extrusion allow enzymatic hydrolysis of the cellulosic polymers.

The Xylan process, by eliminating acid hydrolysis eliminates the production of toxic byproducts or carbonization (acid burning) associated with conventional sulfuric or hydrochloric treatments. The Xylan Delignification Process (XDP) seems also to be simpler and lower cost than the ammonia explosion (AFEX process, Dale et al., 1985) in which a high pressure explosion chambers must be developed, tested and scaled-up. We will follow the XDP process with an enzymatic hydrolysis of the biomass polymers.

The BPI steep delignification process is also a non-acid process, which does not required high pressures or temperatures. We are developing a process which incorporates a high level of chemical recycle within the process. We will be working with the Xylan and BPI steeping process during our follow-on project. Enzymatic hydrolysis is slower than acid or caustic chemical hydrolysis, but does not require the large volumes of chemicals, chemical recovery or neutralization, or the plant to be constructed of special acid/caustic compatible materials. We feel that the mild conditions and low chemical costs associated with enzymatic hydrolysis will outweigh the advantages of chemical (acid or caustic) hydrolysis. There are many different technologies currently under development to convert biomass to ethanol, but despite some rather extensive development efforts, there are no successfully operating biomass to ethanol plants running presently in the US to our knowledge.

E. Lab scale development of a flocculent yeast strain for xylose fermentation

The conversion of biomass to ethanol is being actively pursued by a number of researchers and government programs both in the US and Canada. Ethanol production within the boundaries of the US should allow a direct displacement of imported petroleum used for transportation fuels. There is no real reason why fertile farm states such as Indiana could not produce a large fraction of the liquid transportation fuels needed in the United States on a renewable basis. This project's basic goal is to produce ethanol economically in an environmentally friendly and energy efficient manner from domestic cellulosic biomass sources. Currently, almost all ethanol in the US is made from corn/grain starch sources. There is currently excess grain producing capacity in the US which allows the use of this grain for ethanol production, but in the long run, as export markets improve, it may be more beneficial to use the grains for human consumption. Biomass resources in the US are enormous, and include paper wastes, urban lawn wastes, wood pulp, straw, corn stalks, saw dust, sugar cane bagasse, etc. There is also some current research on production of biomass crops such as switch grass and alfalfa as feedstock for ethanol processes with a recent issue of Biomass & Bioenergy devoted to research papers on dedicated biomass feedstock supply systems in the US (B&B 6:3 1994).

Conversion of biomass to ethanol is difficult due to 1) the resistant nature of biomass to breakdown 2) the variety of sugars which are released when the hemicellulose and cellulose polymers are broken and the need to find or genetically engineer organisms to efficiently ferment these sugars, 3) costs for collection and storage of low density biomass feedstocks.

In this portion of the project, we evaluated different strains for the production of ethanol from xylose, which is the predominant monomer component of hemicellulose. There are three major efforts currently underway in the US to develop genetically engineered yeast (Ho et al, Purdue Univ., W. Lafayette), E. coli bacteria (Ingram et al, U. of Florida, Gainesville), and Z. mobilis bacteria (NREL Golden CO). Each of these groups is developing organisms which are capable of fermenting both glucose and xylose sugars. However, while wishing these groups the best of luck, it is our goal to pursue the use of dual strains of non-genetically engineered yeasts.

Bacterial fermentations generally need to be run at neutral pH's which make these fermentations particularly vulnerable to contamination by other bacteria. Yeasts, on the other hand, are tolerant of low pH fermentations which, along with the addition of some antibacterial oxidants (SO₂, chlorine etc.) allows for the preferred fermentation by the yeast.

The other problem with a single culture is that almost all fermenting organisms prefer glucose to other sugars, leading to a diauxic utilization of sugars, with glucose first consumed, followed by xylose. If significant amounts of ethanol result from the glucose fermentation are present, the fermentation is

slowed first by the presence of ethanol, and second by the difficulty of fermenting xylose through the genetically added metabolic pathway.

We feel as though the most promising near term fermentation system is the dual fermentation process using non-genetically engineered strains of yeast. Therefore, we did an evaluation of xylose fermentations by four strains of yeast as described in Appendix E.

In this study we found that a strain of *Pichia stipitus* seemed to have performance advantages over other strains evaluated. We have since developed a flocculent sub-strain of this organism.

F. Application of the CSRS to biomass hydrolysate in a Dual Culture Multi-Stage CSRS

The successive fermentation of sugars from biomass will be studied in a follow on ERIP project. We suggest separating the fermentations of cellulose (glucose) and hemicellulose (xylose). These fermentations can be performed sequentially on different stages of the CSRS with different fermentation organisms maintained on the two sections of the unit. If the XDP process is utilized, release of xylose will allow the first stages of the reactor to be xylose fermentations, followed by cellulose/glucose SSF in the final stages of the system. This is the process suggested in Appendix D.

If the BPI steep process is used, both sugars are released enzymatically from the treated biomass. The first stages then will be glucose utilization by a *Saccharomyces* strain (Proprietary BPI strain BP-11) followed by xylose fermentation and ethanol stripping in the final stages. Xylose fermentation tends to be a slower fermentation, with specific conversion rate for xylose yeasts about 10 to 25% of the rates given by *Saccharomyces* on glucose.

G. Design of a Solvent Absorption / Extractive Distillation Process for Ethanol Recovery from Vapor Streams

Energy savings for ethanol distillation can be attained by combining the CSRS reactor concept, which strips ethanol into a circulating gas phase, with solvent absorption of the ethanol from the gas stream exiting the CSRS as shown in Figure G.1. The ideal solvent for absorption of ethanol from a gas stream would have the following properties:

- 1) low vapor pressure of the solvent,
- 2) solvent miscible with ethanol
- 3) solvent vapor carry-over non-toxic to fermenting microbes in CSRS,
- 4) low solubility of water in the solvent,
- 5) low solubility of solvent in water.

The ideal solvent would absorb only ethanol allowing an anhydrous ethanol product to be stripped off from the solvent/ethanol stream from the absorber. Combining the ethanol enrichment of the ICRS or CSRS (vapor

19a

Figure ..The Solvent Absorption-Extractive Distillation (SAED) process for ethanol recovery from gas streams.

concentration of 5 to 8 times higher than liquid) with the enrichment of ethanol in the solvent absorber (limited water solubility in the solvent) allows highly concentrated ethanol to be easily recovered from the solvent in a solvent stripping column. As the solvents currently under consideration are 'high boilers' only trace amounts of solvent vapor enter the CSRS, and based on studies to date with an immobilized cell reactor, these vapors leave the reactor at the same concentration as they enter.

Over the course of this project, a number of solvents were evaluated for their selectivity, α , where $\alpha = K_{de} / K_{dw}$, and:

$$K_{de} = C_{e(s)} / C_{e(aq)}, \quad K_{dw} = C_{w(s)} / C_{w(aq)} \quad \text{Eq. 9}$$

where $C_{e(s)}$ is the concentration (wt %) of ethanol in the solvent etc. The design and performance of a pilot scale SAED system are described in Appendix H, and a full scale SAED system was designed, built, and installed at the PRI site for ethanol recovery from the 24,000 L CSRS as shown in Figures H. 6 and H. 7.

H. Design, Construction, and Performance of a 24,000 L. CSRS- SAED system

A 24,000 liter, 4 stage Continuous Stirred Reactor- Separator (CSRS) was designed and built with the support of this project, the Great Lakes Governors, and Permeate Refining Inc. The reactor separator is coupled with a solvent absorber for ethanol recovery from the gas stream. The Solvent Absorber/ Extractive Distillation system should allow re-use of the solvent as a extractive distillation agent, making water more volatile than ethanol. Water can thus be distilled off the oil/ethanol mixture, and the anhydrous ethanol then stripped from the oil. A schematic of the CSRS unit is shown in Figure H.1, and a simplified schematic of the flows in the SAED is shown in Figure G.1. Designs and photographs of the installed CSRS (H. 2, H. 3, H. 4 and H. 5). and SAED (H. 6 and H. 7) follow. Details of the unit flows and performance as a cascade fermenter are given in Appendix H. The CSRS was constructed between September '94 and April of '95, installed in May of 1995, and started in June. It has been operated continuously as a cascade reactor system between June of '95 through March of 1997 except for brief shut-downs to fix or modify the system. The unit was operated primarily for the first year on candy rinse water from a Chicago area candy manufacturer, and primarily since September 96 to the present on hydrolyzed waste starch from Cedar Rapids, IA and Hammond IN.

Figures H. 8 and H. 9 show fermentation performance of the unit over a period of 6 months using a highly flocculent yeast strain developed as part of this project. This performance is also discussed in Appendix H.

NOTE:

ALL MATERIAL SHALL BE 304 STAINLESS STEEL

SCALE

ALCOHOL DISTILLATION TOWER PROJECT

towsec (TOWER ASSEMBLY SECTION VIEW)

3/14/94

BKing

2/28/94

ITEM	DESCRIPTION	DATE
1	TANK BAFFLE	D1

H3

SECTION "B-B"

Figure H.3 A Design of a CSRS Stage

NOTE: ALL MATERIAL SHALL BE 304 STAINLESS STEEL

FILE	Alcohol Distillation Tower Project	SCALE	DATE
			3/14/94
DRAWING NAME	sec1 (TOWER SECTION 1)	BY	BKing
		DATE	2/23/94

Figure H.2. A Photograph of the 24,000 L CSRS at the PRI site (vertical-looking down)

20d

Figure H. 4 A photograph of a CSRS Stage

20 e

Figure H. 5. A Photograph of a CSR sieve tray separation stage

HS

Figure H. 6 A Design Schematic of the the SAED system at the PRI site

Figure H. 8. A Photograph of the installed SAED system at the PRI site.

FEB - JUNE PERFORMANCE

Figure H. 9 Performance of the CSRS as a Cascade Reactor for 9/95 through 1/96

Figure H. 10 Performance of the CSRS as a Cascade Reactor for 2/96 through 6/96

3. Conclusions and Suggestions for Further Research

The basic goal of this project was to develop and demonstrate the CSRS concept for the fermentation of starch, whey permeate, permeate mother liquor, molasses, or cellulosics, with the intent of developing a fermentation process which would allow smaller scale, low energy, economical conversion of various substrates to anhydrous ethanol.

We were able, over the extended period of this ERIP project (1994-1997) with the combined support of several other sponsors (as listed on the coverage of this report) able to:

- 1) build and operate a 4 liter CSRS on sugars, converted starch, raw starch, and molasses,
- 2) develop, model, and screen solvents for the SAED system,
- 3) build and test a two column 80 liter pilot scale SAED system,
- 4) build and operate a 24,000 Liter CSRS,
- 5) test and screen flocculent yeast for the high speed fermentation of glucose, sucrose, molasses, and fructose,
- 6) test and screen yeasts for xylose fermentation,
- 7) test the Xylan pretreatment process for its ability to allow enzymatic fermentation of cellulosics,
- 8) build a 75 Liter 6 stage CSRS for cellulosics fermentation,
- 9) begin development of a proprietary steeping pretreatment process, and
- 10) build a two column SAED system to accompany the 24,000 Liter CSRS system, 11) evaluate the used of cascade and CSRS type fermenters for the fermentation of Permeate Mother Liquor (PML),
- 12) work with a Minnesota ethanol producer on the continous fermentation of PML at an industrial site.

We are currently negotiating with a small company , Eco-Products Corp., for the first commercial installation of a CSRS/ SAED system. The Eco-Products Corp. of Indiana is planning to install a 2 to 3.5 million gallon per year ethanol plant using candy and waste carbohydrates from Chicago area food processors. We also plan to promote this technology in Wisconsin for whey lactose and PML conversion to ethanol.

Thus we feel that the ERIP program has successfully helped BPI move this technology from concept to practice, being quite optimistic about the near-term implementation of the technology at a number of mid-western US sites.

Suggestions for Further Research

A follow-on project supported by the ERIP for application of the technology to cellulose is just starting, with Fred Hart serving as project coordinator. This project should help move the technology towards a further substrate, biomass from wood, corn stalks, waste paper, and straw.

The use of the technology on raw starch should be pursued. Our results were quite positive, and a small feed lot type location would be an ideal type situation for a raw starch operation as shown in Figure 3.1

Further screenings/ development of solvents could possibly reduce energy requirements for the production of anhydrous ethanol.

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Appendix A.

Osmotic Inhibition of Free and Immob K.marx
Dale et al (1994)

Osmotic Inhibition of Free and Immobilized *K. marxianus* Anaerobic Growth and Ethanol Productivity in Whey Permeate Concentrate

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The effects of high concentrations of whey permeate on a lactose fermenting yeast, K. marxianus, were studied using both free and immobilized cell reactors. Examination of substrate and product inhibition data from the literature suggests that a simple linear osmolality model may account for both the substrate and product inhibition of anaerobic yeast growth and ethanol productivity for this yeast and others. The osmolality of sweet whey permeate concentrate (SWPC) and acid whey permeate concentrate (AWPC) solutions were determined and fitted to a simple empirical model. The batch free cell growth rates and immobilized cell growth and productivity in these solutions was then determined. Batch cell growth was found to be 85% inhibited at osmolalities of 2.2 os/kg in both AWPC and SWPC, although the maximal specific growth rate for SWPC was 2.6 times higher than for AWPC. Immobilized cell growth inhibition patterns were different between AWPC and SWPC. An 80% inhibition in effluent cell density was noted at an osmolality of 1.3 os/kg with AWPC, while with SWPC, this level of inhibition was not reached until 1.9 os/kg. The cell growth of the immobilized cells was more strongly inhibited than the free cells at higher solution osmolalities.

The effect of osmolality on immobilized cell productivity as measured by gas evolution rates was also examined. A 66–85% inhibition of productivity was noted at 2.4 os/kg (31% solids) with AWPC, while SWPC was only 30–60% inhibited at 2.2 os/kg (40% solids). The immobilized cells were more tolerant to high osmolalities if the osmolality was increased slowly over time, indicating adaptation by the immobilized cells. The productivity of immobilized cells was less inhibited by high osmolality than free cell productivity as reported by other researchers.

NOTATION

K Numerical constants
 K_{ev} Linear constants for osmotic inhibition of productivity (osmol/kg)

K_{μ} Linear constant for osmotic inhibition of cell growth (osmol/kg)
 P Product concentration (ethanol) (g/litre)
 P_{max} Product concentration (ethanol) where growth or productivity is totally inhibited (g/litre)
 S Substrate concentration (lactose, sucrose) (g/litre)

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S_0	Threshold sugar concentration where osmotic inhibition is first noted (g/litre)
x_s	Percentage solids (w/v) in solution
ϵ_0	Initial osmolality at which inhibition is first noted (osmol/kg)
μ	Specific growth rate (g cell/g cell h)
$\mu_{\max}$	Maximum (uninhibited) specific growth rate (g cell/g cell h)
ν	Specific productivity (g eth/g cell h)
$\nu_{\max}$	Maximum (uninhibited) specific productivity (g eth/g cell h)

INTRODUCTION

Each organism has a maximal, optimal, and minimal water activity for growth and productivity. In general, microbes will tolerate a lower minimal water activity if all other environmental parameters (temperature, pH, nutrient mix) are near optimal for that organism. Most bacteria and yeast have a growth limiting minimal a_w of around 0.91 (5.2 os/kg). But osmophilic yeasts can grow slowly in media with a_w s as low as 0.62–0.65 (23.9–26.5 os/kg).^{1,2} If the osmotic environment of a solution is higher than the natural internal osmotic pressure within the cells, there will be a net flux of water from the cell. The osmolality of a solution is an additive property of the osmolality of the various solutes (assuming that solute interaction effects are small), which makes osmolality a useful function to work with in fermentation systems. The osmolality of a glucose solution being fermented to ethanol increases as one mole of sugar is converted to two moles of ethanol. Jones and Greenfield¹ note that in batch yeast fermentations exponential growth cycle cessation was often noted at a point in the fermentation where solution osmolality began to increase.

Glycerol is used by *Saccharomyces cerevisiae* yeast as an osmoregulatory internal product. The osmolality inside the cell is regulated to be slightly higher than the external environmental osmolality. Under conditions of minimal a_w environments (maximal solution osmolality) glycerol production in the cell can not keep up with the leakage rate through the cell membrane, plasmolysis results and there is not cell growth.³ Kenyon *et al.*⁴ noted a fourfold increase in glycerol levels when a CSTR was operated at an osmolality calculated as 1.6 os/kg versus a 0.33 os/kg solution. Osmotolerant yeasts have a higher natural internal osmotic

potential (3.5 os/kg for *Saccharomyces rouxii*) allowing higher osmotic environments to be tolerated before osmotic inhibition starts. Osmotolerant yeast also seems to be less permeable to polyols and thus better able to retain these internal osmoregulatory products, with the major polyol produced by *S. rouxii* being arabitol rather than glycerol.⁵

The onset of osmotic inhibition of ethanol productivity for yeast seems to begin at osmolalities above the natural internal osmolality of the cell. In a study with two strains of *S. cerevisiae*, osmotic inhibition of growth began at an osmolality of 1.0 for both strains tested, falling linearly to zero at an osmolality of 3.0 for the more osmotolerant strain while the less tolerant strain showed plasmolysis at slightly under 2.0 os/kg.⁶ With *Saccharomyces uvarum* yeast, a 400 g/litre sucrose solution (2.4 os/kg initial osmolality) was noted to inhibit maximum batch productivity by 62% as compared to a 250 g/litre sucrose solution (initial osmolality of 1.07 os/kg). Altering the osmotic environment of a 10% sucrose solution in a non-growth medium with increasing levels of sorbitol gave maximum ethanol productivity in a fermentation broth with an initial osmolality of 0.63 and final osmolality of 1.37. The total ethanol productivity was reduced by 46% and the final cell viability reduced by 80% in a fermentation with an initial osmolality of 2.9 and final osmolality of 3.3 os/kg.⁷ It was also noted that cells retained ethanol internally for longer periods of time during batch fermentations and showed a higher final inter-cellular ethanol concentration as the osmolality of the environment increased. Another investigation of osmotic influences on yeast using an 18% glycerol solution to give an initial broth osmolality of 2.84 showed a 54% decrease in productivity at 6 h as compared to a solution with an initial osmolality of 0.78.⁸

In a recent study on the effects of osmolality (using glycerol to modify the osmotic potential) on free and immobilized *Zymomonas mobilis* at a high operational temperature,⁹ it was determined that immobilized cell growth was more strongly inhibited than that of free cells by increasing osmolality, but that the same inhibition pattern was noted. It was postulated that the immobilized cells, due to their high concentrated density on the column packing, might be experiencing an osmolality of 1.5 os/kg higher than the bulk solution. The productivity of the immobilized cells was slightly more inhibited than the free cells at osmo-

lalties of under 5.0 os/kg, but the immobilized cells showed higher productivity than free cells at osmolalities over 6 os/kg. Ethanol inhibition of cell growth and productivity was much stronger than inhibition due to only solution osmolality, suggesting specific ethanol inhibition at 40°C for this organism.

If new 'plug flow' type immobilized cell reactor-separators implementing simultaneous production separation are used, ethanol product inhibition limits are lifted allowing more concentrated feeds to be fed to the reactor.¹⁰⁻¹² The new limiting factor on maximum feed concentration to the bioreactor becomes the ability for the fermenting organism to withstand the high osmotic potential of the feed. If the fermentation of high concentrations of substrate is desired, it will be critical to know the effects of high solution osmolality on cell growth and productivities. If growth rates are more inhibited than productivities by high osmolalities, feed concentration to a bioreactor might be increased to limit cell growth and the concomitant reactor clogging in immobilized cell type reactors. Reactor clogging by cell overgrowth can be a problem in trickle flow type immobilized reactors.^{11,12} Osmotic inhibition may also be rate limiting for stirred tank type reactors incorporating simultaneous ethanol removal. Feeds such as molasses and whey have substantial mineral components, and these build up in the reactor. Cysewski and Wilke,¹³ show that a certain rate of bleed is required to keep the non-fermentables in the 'vacu-ferm' reactor contents from building up to the point where osmotic inhibition reduced reactor performance. In the similar concept 'Biostil' reactor¹⁴ (where a side stream from the reactor is stripped of ethanol and returned) osmotolerance of the yeast, rather than ethanol tolerance is most critical to reactor performance. The objective of this investigation was to determine the effect of high concentrations of whey permeate, both sweet and acid types, on cell growth and ethanol productivities of a lactose fermenting yeast using batch and immobilized cell reactors.

Certain strains of lactose utilizing yeasts have been screened and determined to be more tolerant to high levels of feed lactose. Moulin *et al.*¹⁵ selected two strains as being able to ferment 30% permeate with 50 and 55% of the lactose being converted to ethanol. Vienne and Stockar¹⁶ showed that strain *Kluyveromyces marxianus* 665 was able to ferment an 18% whey permeate solu-

tion faster and with a slightly higher efficiency than other strains, including the strains used by Moulin *et al.*¹⁵ Strain *K.m.* 665 was selected for this research.

MATERIALS AND METHODS

Strains and permeate

K. marxianus NRRL 665 was obtained from the Northern Regional Research Laboratory in Peoria, IL. Dried sweet whey permeate was purchased from Wisconsin Dairies Coop, concentrated sweet whey permeate was provided by Swiss Valley Farms of Maquoketa, IA, and concentrated acid whey was provided by Dairy Farms Co. of Orville, OH.

Determination of osmolality

A vapour pressure osmometer, Westcor model 5100CXR, was used for the determinations of osmolalities of molasses solutions. This osmometer is able to determine osmolalities of solutions up to a maximal determination of about 3.5 os/kg. Whey permeate solutions were tested, and lactose solutions were also prepared to check the reliability of the readings as well as to obtain osmolalities beyond those given in reference texts.¹⁷

Batch studies

Flasks or tubes of various concentrations of reconstituted dried whey permeate, concentrated sweet whey permeate, and concentrated acid whey permeate were prepared. An inoculum of 1% of aerobically grown cell suspension was added to each batch reactor. These were grown in still culture with the cell density determined using the optical density at 660 nm as a function of time.

Immobilized cell reactor (ICR) studies

A short 'differential' type columnar reactor was constructed of a 2 in ID glass tube packed with a piece of terry cloth and spiral wound with a nylon spacer (Fig. 1). The reactor was run in a trickle bed, gas continuous mode. It had an internal packed volume of 115 ml, and a liquid hold-up of 30 ml. The feed rate to the reactor was held constant at 60 ml/h for a liquid residence time of 30 min. *K. marxianus* 665 was grown in a flask of sterile nutrient medium (10 g/litre lactose, 3 g/litre yeast extract, and 3 g/litre malt extract). After 24 h growth in this medium, the yeast broth

lalties of under 5.0 os/kg, but the immobilized cells showed higher productivity than free cells at osmolalities over 6 os/kg. Ethanol inhibition of cell growth and productivity was much stronger than inhibition due to only solution osmolality, suggesting specific ethanol inhibition at 40°C for this organism.

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A vapour pressure osmometer, Westcor model 5100CXR, was used for the determinations of osmolalities of molasses solutions. This osmometer is able to determine osmolalities of solutions up to a maximal determination of about 3.5 os/kg. Whey permeate solutions were tested, and lactose solutions were also prepared to check the reliability of the readings as well as to obtain osmolalities beyond those given in reference texts.¹⁷

Batch studies

Flasks or tubes of various concentrations of reconstituted dried whey permeate, concentrated sweet whey permeate, and concentrated acid whey permeate were prepared. An inoculum of 1% of aerobically grown cell suspension was added to each batch reactor. These were grown in still culture with the cell density determined using the optical density at 660 nm as a function of time.

Immobilized cell reactor (ICR) studies

A short 'differential' type columnar reactor was constructed of a 2 in ID glass tube packed with a piece of terry cloth and spiral wound with a nylon spacer (Fig. 1). The reactor was run in a trickle bed, gas continuous mode. It had an internal packed volume of 115 ml, and a liquid hold-up of 30 ml. The feed rate to the reactor was held constant at 60 ml/h for a liquid residence time of 30 min. *K. marxianus* 665 was grown in a flask of sterile nutrient medium (10 g/litre lactose, 3 g/litre yeast extract, and 3 g/litre malt extract). After 24 h growth in this medium, the yeast broth

Fig. 2. Relative productivity of *C. pseudotropicalis* as a function of osmolality on lactose and ethanol (data from Moulin *et al.*¹⁵).

Fig. 4. Relative growth rates of *S. cerevisiae* as a function of osmolality (data from Letourneau and Villa¹⁸).

Fig. 3. Relative productivity of *C. pseudotropicalis* as a function of osmolality on glucose and ethanol (data from Moulin *et al.*¹⁵).

similarly transformed as shown in Fig. 4 with 0 os/kg for ϵ_0 and 2.0 os/kg for $K_{\epsilon\mu}$. Thus, both substrate and ethanol inhibition of growth and productivity are largely the osmotic phenomena for these yeast strains. Table 1 shows the correlation coefficients (r^2) between the linear osmolality model and the more complex (up to five coefficients) models proposed by Moulin *et al.*¹⁵ and Letourneau and Villa.¹⁸ The fit by the simple linear osmolality model is nearly as good in all cases.

The results shown here suggest that both ethanol (product) and sugar (substrate) inhibition of anaerobic yeast cell growth and productivity are

largely osmotic phenomena. This observation should be helpful in the design and operation of ethanol reactors. The inhibition effects of inerts (salts, glycerol, lactic acid, and other non-fermentables) can be substantial with complex substrates like molasses, whey (both sweet and acid types), and in reactors in which broth is evaporated or recycled.^{13,14} Osmolality in such a reactor can be estimated if the composition of sugars, ethanol and inerts is known (allowing the osmolality to be determined), or the osmolality of the reactor may be monitored as a controlled variable even if the composition of inerts is unknown or complex. Measuring or estimating the solution osmolality will give a good prediction of the expected cell growth and productivity inhibition due to inerts, substrate and product.

There may, of course, be further toxic or inhibitory compounds in the broth with specific effects beyond osmotic. Ethanol has further toxic effects on yeast besides its osmotic inhibiting effects. The effects of ethanol on various yeast species has been examined by a number of researchers, and many models for ethanol inhibition of yeast suggested. The fact that (1) ethanol inhibition of cell growth and productivity increases with increasing temperatures, and (2) ethanol increases the cell death rate, are evidence that ethanol has toxic effects on the yeasts beyond osmotic inhibition. The toxic effects of ethanol (as distinguished from the osmotic inhibition effects) are particularly important for immobilized cell reactors, as Dale *et al.*^{19,20} show that the live cell fraction of a dynamic immobilized cell population can be expected to

Table 1. Osmolality model for substrate and product inhibition of yeast

	Model	Solution	r^2
Proposed	$v/v_{\max} = \left[1 - \frac{\varepsilon - \varepsilon_0}{K_{\varepsilon v}} \right]$ $K_{\varepsilon v} = 2.3$ $\varepsilon_0 = 0.5$	Lactose and ethanol ¹⁵	0.929
Moulin <i>et al.</i> ¹⁵	$v/v_{\max} = \exp(-K_{L1}P)$ $-K_{L1} = 0.0157$	Lactose and ethanol ¹⁵	0.924
Proposed	$v/v_{\max} = \left[1 - \frac{\varepsilon - \varepsilon_0}{K_{\varepsilon v}} \right]$ $K_{\varepsilon v} = 3.1$ $\varepsilon_0 = 0.5$	Glucose and ethanol ¹⁵	0.927
Moulin <i>et al.</i> ¹⁵	$v/v_{\max} = \exp(-K_{G1}P) \exp(-K_{G2}(S - S_0))$ $\exp[-K_{G3}(S - S_0)P]$ $K_{G1} = 9.4 \times 10^{-3}$ $K_{G2} = 3.5 \times 10^{-3}$ $K_{G3} = 1.9 \times 10^{-4}$ $S_0 = 100 \text{ g/litre}$	Glucose and ethanol ¹⁵	0.969
Proposed	$\mu/\mu_{\max} = \left[1 - \frac{\varepsilon}{K_{\varepsilon \mu}} \right]$ $K_{\varepsilon \mu} = 2.0$	Beet molasses and ethanol ¹⁸	0.950
Letourneau and Villa ¹⁸	$\mu/\mu_{\max} = \exp(-K_{S1}(S - S_0)) \left[1 - \frac{P}{P_m} \right]^{(K_{S2} + K_{S3}S)}$ $K_{S1} = 5.5 \times 10^{-3}$ $K_{S2} = 0.4$ $K_{S3} = 7.4 \times 10^{-3}$ $S_0 = 117 \text{ g/litre}$ $P_m = 70 \text{ g/litre}$	Beet molasses and ethanol ¹⁸	0.959

decrease over time if growth rates are not higher than cell death rates.

Whey permeate osmolality

The osmotic potentials of various whey solutions were determined as shown in Fig. 5. Whey permeate has a higher osmolality than lactose, due to the salt fraction, and AWPC has a higher osmolality than SWPC. Acid whey with a pH of 4.5 is a by-product of cottage cheese production, and has more lactic acid than sweet type whey which has a pH of 5.3–5.5. Reconstituted dried SWPC was found to demonstrate a higher osmolality than

fresh SWPC at the same solids concentration. The reason for this difference in osmolality may be due to precipitation of some proteins and/or higher molecular weight salts in the drying process. Lactose osmolality to 24% solids (the solubility limit at room temperature) was determined. Our determinations at lower concentrations were close to literature values¹⁷ as shown in Fig. 5.

The data on solution osmolality as a function of solids were fitted empirically to a simple two parameter model based on percentage solids (x_s):

$$\varepsilon_s = [k_1 x_s]^n \quad (4)$$

Fig. 5. Osmolality of various whey permeate solutions as a function of percentage solids. (Dotted lines show the fit of the data to eqn (8) using the constants from Table 2.)

Parameters k_1 and n were determined as shown in Table 2 for the various solutions and the resulting fits are shown in Fig. 5 as the dotted lines.

Batch growth studies

Six replications of batch growth curves were performed for acid whey permeate at concentrations of 15, 20, 25 and 30% solids, and sweet whey permeate at 15, 20, 25, 30, 35 and 40% solids. Sample growth curves are shown in Figs 6 and 7, with the averaged maximal exponential specific growth rates plotted against osmolality in Figs 8 and 9. It can be seen that the growth rates are very inhibited above an osmolality of 2.0 with both sweet and acid whey showing a total inhibition of growth at about 2.4 os/kg if the inhibition curve is extrapolated to zero growth rate. The maximal specific growth rate with sweet whey (at 15% solids) was 2.6 times higher than AWPC (μ of 0.396 h^{-1} for SWPC versus 0.152 for AWPC).

ICR cell growth and productivity

The productivity of a short differential immobilized cell reactor as a function of different feed concentrations was next determined. Cell densities in the reactor effluent were also monitored as an indicator of immobilized cell growth. If a steady state cell density in the reactor is assumed, then the effluent cell density can be used to estimate the number of cells grown.²⁰ The effects of AWPC concentration and osmolality on effluent cell density and relative productivity are shown in Figs 10 and 11. Cell growth of the immobilized cells was inhibited at 20% solids (1.34 os/kg), and there was no major difference in growth inhibition between when osmolalities were increased or

Table 2. Osmolality constants for various solutions (eqn (4))

	k_1	n	r^2
Lactose	0.040	1.20	0.985
Sweet whey permeate concentrate (SWPC)	0.046	1.33	0.993
Reconstituted sweet whey permeate concentrate (RSWPC)	0.053	1.32	0.990
Acid whey permeate concentrate (AWPC)	0.058	1.32	0.999
Glycerol	0.121	1.23	0.998

Fig. 6. Batch growth of *K. marxianus* in concentrated acid whey permeate at various concentrations.

Fig. 7. Batch growth of *K. marxianus* in concentrated sweet whey permeate at various concentrations.

decreased with time. An 85% drop in effluent cell densities was noted as the feed concentration increased from 15 to 20% (0.92–1.34 os/kg) with little further inhibition as the osmolality was increased. Productivity was less inhibited than growth at higher osmolalities. There was a major difference in the degree of productivity inhibition when osmolality was increased with time rather than decreased. When the osmolality was

Fig. 8. Maximal batch growth of *K. marxianus* as a function of acid whey permeate concentration ($\mu_{\max} = 0.152$).

Fig. 9. Maximal batch growth rates of *K. marxianus* as a function of sweet whey permeate concentration ($\mu_{\max} = 0.396$).

Fig. 10. Effluent cell density from the ICR as a function of acid whey permeate concentration.

Fig. 11. Relative productivity of ICR as a function of acid whey permeate concentration.

increased from 0.92 to 2.4 slowly over the period of a week, the productivity dropped gradually to 35% of the original rates. But, when the osmolality was changed abruptly from basal 0.92 os/kg to 2.4 os/kg, productivity at 2.4 os/kg was only 13% of the basal rate.

With SWPC, effluent cell densities showed a difference in inhibition patterns between increasing and decreasing osmolality (Figs 12 and 13). The effluent cell density decreased fairly linearly between 1.3 and 1.8 os/kg when osmolality was increased with time. When osmolality decreased with time, a steep decline in effluent cell density was noted between 1.1 and 1.3 os/kg. Productivity patterns for SWPC were fairly similar when concentration increased or decreased with time, with much less inhibition noted at higher osmolalities than with AWPC. Productivity showed only a 15% decrease at 1.9 os/kg when concentration increased or decreased with time, inhibition was noted at osmolalities under 2.0 os/kg.

The effects of osmolality on the productivity of immobilized cells in AWPC and SWPC are compared to the effects noted by Moulin *et al.*¹⁵ with free cells in a lactose-ethanol medium in Fig. 14. We see that the immobilized cells in SWPC seem to be less inhibited than the free cells, probably due to adaptation of the immobilized cells. The cells in the acid whey are more strongly inhibited by increasing osmolality than with the SWPC, indicating that there is some constituent in the AWPC which is inhibitory to the yeasts. The decreased productivity rates seen in concentrated AWPC are not due solely to osmotic effects. As acid whey is produced by the lactic acid ferment-

Fig. 12. Effluent cell density from the ICR as a function of sweet whey permeate concentration.

Fig. 13. Relative productivity of ICR as a function of sweet whey permeate concentration.

tation of milk during the cheese making process, it seems likely that there is some low level by-product(s) of the lactic acid fermentation that are inhibitory to yeast, such as acetic or propionic acids, and flavour compounds (diacetyl, acetaldehyde and acetoin). Volatile acids (acetic, propionic) generally account for about 2–3% of the total acid produced.²¹ There is no doubt that the lactic acid fermentation of milk to cottage cheese or yogurt greatly improves the 'keeping' qualities of the fermented product, indicating the formation of inhibitory compounds. Lactic acid itself is not particularly inhibitory at the levels found in AWPC (5–20 g/litre) based on preliminary experiments performed in our laboratory (unpublished data).

Fig. 14. Relative productivity of immobilized cells in AWPC and SWPC as a function of osmolality.

CONCLUSIONS

The use of reactors incorporating product separation or the development of ethanol tolerant yeast strains will allow highly concentrated feeds to be fed to a bioreactor. In determining the effects of concentration or feed osmolality on yeast performance, it was determined that both substrate and product inhibition can largely be attributed to osmotic effects, with a linear decrease in productivity and growth noted as solution osmolality increases. Immobilized cells show less inhibition of productivity but higher inhibition of growth with increasing osmolality as compared to free cells. The growth of both free and immobilized cells is much more strongly inhibited than productivity by osmolality. Strong (80%) inhibition of free cell growth was noted at about 2.2 os/kg in both types of permeate, while strong inhibition of immobilized cell growth rates was noted at 1.3 os/kg with AWPC, and in SWPC at 1.8 os/kg. Free and immobilized cell growth and productivity were all significantly lower in acid type whey permeate concentrate as compared to sweet type whey permeate concentrate at the same solution osmolality.

In an immobilized cell reactor, excess cell growth can cause clogging, but lack of any cell growth will lead to reactor performance deterioration as cells die and are not replaced.²⁰ If a maximal concentration of feed is defined as that concentration which gives not more than 80% inhibition of cell growth and not more than 50% inhibition of productivity, a maximal feed concentration of sweet whey permeate concentrate of 35% can be determined, while for acid type WPC

a maximal feed concentration of 20% can be determined. The limits of feed concentrations for both feeds are due to cell growth rather than productivity limitations.

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Appendix . B.

High Density Ferm. w/ Flocc. Yeast
Dale & Zhao (research rpt)

High Density Yeast Fermentations with Flocculent Yeasts

Internal Research Report: 10/95

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Abstract

Four strains of highly flocculent yeast for ethanol production from glucose/sucrose were obtained based on literature references. Each of these strains was evaluated for flocculent behavior and fermentation characteristics. Repeated batch fermentations were performed with settled cells reused ten times. Cell density built up to 22-25 g/l for each of the strains, allowing a 24 hour fermentation of a 20% glucose solution. Actual flocculation and fermentation performances were found to be fairly similar among all four strains, but one strain, *S. cerevisiae* BP-11 was chosen for further investigations as it produced slightly more ethanol than the other strains. Flocculation performance of strain BPY-11 as a function of Reynolds number in a 2 Liter stirred fermenter was studied. A Reynolds number of 50,000 was required to keep the cells in suspension in small flakes. At this Reynolds number there were few (0.2 g/l) single or un-flocculent yeasts, with the yeast observed to settle in a period less than a minute when the agitator was stopped. At a Reynolds number of 180,000, the turbulence was found to cause the de-flocculation of the yeast. After 16 hours, no large yeast flakes remained, with no settling of cells noted within 5 minutes of stopping the impellers. An interesting phenomena was noted in that the yeasts did not re-flocculate after the Reynolds was dropped to 50,000 over a period of 3 days.

Introduction

The rates of ethanol production during a fermentation can be increased more or less linearly by increasing the density of active live yeast or bacteria in a reactor. During a normal batch ethanol fermentation with *S. cerevisiae* a final cell concentration of between 1.5 and 15 g/l cells is achieved. It is often noted that cell growth completely stops after a certain cell density is reached (Holzberg et al, 1967). The oxygen tension in the fermentation is important in these batch fermentations, as the cells will move towards biomass production as the amount of oxygen available to the cells increases. Trace oxygen can serve as a nutrient during the anaerobic fermentation of sugars, allowing the fermentation rate to increase with more cells produced. Cysewski and Wilke (1978) show an optimal oxygen tension of about 0.1 mm O₂. To maintain a cell density higher than the natural maximum attained in the fermenter, methods for keeping the cells in the fermenter must be utilized. A high cell density can be maintained either by recycling cells (through membrane or centrifugal techniques) or by retaining or

immobilizing the cells within the reactor. Immobilization would seem to be advantageous as the capital expense of a cell recovery and recycle system can be eliminated. There has been a good deal of work over the last 10-15 years on immobilizing organisms to maintain a high cell density in the bioreactor. Immobilization can take one of several approaches, 1) entrapment within a gel bead or plate, 2) adsorption onto a solid matrix, or 3) self-agglomeration or flocculation into flakes or pellets.

1) gel entrapment- A large body of literature is available on lab and pilot scale attempts to immobilize cells within gel beads or sheets. These have had varying degrees of success, but longevity of the gel bead or sheet is a question which has not satisfactorily been addressed in the scale-up of these sorts of systems. Gas evolution by the fermenting cells has a disruptive effect on the cell matrix. Diffusion of sugar into the bead/sheet and ethanol from the bead are mass transfer inhibitions to achievable fermentation rates. Some studies have shown that cell growth occurs very near the surface of the bead.

2) adsorption onto a solid matrix- Our lab's previous work has focussed on adsorption of cells onto a fibrous matrix (Dale et al, 1987, 1991), run in a gas continuous mode. However, this sort of system is not tolerant to insoluble solids. Cells can also be adsorbed into porous glass or ceramic beads/matrices.

3) self-agglomeration/flocculation- The use of flocculent yeast flakes or pellets for yeast fermentation has been suggested by several researchers. We felt that this system is the simplest method for maintaining a high cell density in the reactor. Some yeast have the property of joining together in clumps or flocs, with these multi-cell clumps having a much more rapid settling velocity than single cells. Flocculation is an important factor in the brewing process. After the beer or wine has completed its fermentation, it is desirable to have the yeast settle out. Standard *S. cerevisiae* used for beer and wine fermentations are selected to have this post-fermentation flocculation characteristic. Standard wine or champagne yeast settle over a period of 150 to 300 minutes if there is no fermentation activity to suspend the cells (Arikan and Ozilgen, 1992), while flocculent cells tend to settle so quickly it is difficult to get an OD on the cells as the cells settle in one minute in a cuvette (Castellon and Menawat, 1990). There is a body of literature on flocculation available with a review of the literature available (Calleja, 1989) and a number of papers discussing the effects of sugars (Kihn et al, 1988), ions (specifically sodium as a deflocculant (Castellon and Menawat, 1990) and calcium as a pro-flocculant (Kihn et al, 1988a; 1988b; and Masy et al, 1990). A microscopic study of flocculating fission yeast was reported by Sowden and Walker (1987, 1989) where a "hairy" or "mucilaginous" coating of the yeast is described. Soares et al, 1992 show that cell-cell interaction is important with a cell suspension of 2×10^7 cell/ml reaching a free cell density of 0.5×10^6 cell/ml in 2 minutes while a suspension starting at 1×10^7 cell/ml also settled to this same free cell density in 2 minutes.

The use of highly flocculent yeast for continuous reactors has been demonstrated by APV in a tower fermenter. Cell densities of 70-80 g/l were reported (Greenshield and Smith, 1970). Chen and Gong (1986) did further work on a flocculent tower type reactor, while Cysewski and Wilke (1977) used a simple settler with cell recycle to attain high cell densities in a stirred reactor.

To begin our experiments, literature descriptions of highly flocculent yeasts were used to select four strains which were kindly provided by C.L. Kurtzman of the NCAUR in Peoria, IL. These strains were 1) *S. cerevisiae* BPY- 11. 2) *S. bayanus* BPY 12 3) *S. cerevisiae* BPY-13. 4) *Schizos. japonicus* BPY- 14

Methods and Materials

The yeast were grown micro-aerobically on a 50 g/l glucose medium supplemented with 3 g/l each of yeast extract, malt extract and peptone (YMP). Each 250 ml flask was filled with 150 ml of fermentation media. In subsequent repeated batch fermentations, the cells were allowed to settle for 15 minutes, after which the supernate broth was poured off, and a 22% glucose YMP media returned to the cells to bring the fermenter volume back to 150 ml. The flasks were held in an incubator at 30 C, and stirred with a magnetic stirrer at 180 RPM. Following the 10th consecutive fermentation, the settling/flocculation performance of the strains was compared.

Flocculation was measured by the settling speed as determined using a method developed in our lab. This method consists of filling a 50 ml graduated test tube with cell broth and observing the settling behavior over time, and taking samples of the the two layers. As time passes, the suspension divides into two layers, a supernate or top layer, and a cell concentrate (sedimenting) or bottom layer. The speed at which these layers form is a measure of the settling speed of the yeast. Kihm et al (1988) suggested measuring the OD of the supernate left in a tube after 5 minutes as being a measure of the flocculating nature of the yeast with the lower the OD the greater the degree of flocculation, but we feel our method gives a more accurate description of the settling behavior of the yeast floccs.

Results

Flocculation Characteristics

After repeated batch fermentations using the various flocculating strains, the flocculation characteristics of each of the strains was noted. A clear 50 ml tube was filled with fermentation media, and observed as it separated into a supernate and sediment layer. The concentration of yeast in the sediment layer is shown in Figure 1. It can be seen that the sediment layer begins at a concentration of about 25 g/l and slowly compresses to a concentration of 70 to 85 g/l. Strain BPY-12 seems to compress more rapidly than the other 3 strains, but otherwise, similar performance was shown by each of the strains. The concentration of the supernate over time for each of the strains is shown in

Figure 2. Suspended cell concentration in the supernate drops from 7.5 to 3.8 over the first 8 minutes for strain BPY-13. Strain BPY-14 drops more slowly from 5 g/l to 3 g/l over 15 minutes. Strains BPY-11 and BPY-12 seem to be more completely in flocculant form, with the suspended cell concentration of BPY-11 dropping to under 2 g/l in 8 minutes, while the suspended cell concentration of strain BPY-12 is almost immediately under 2 g/l. Figure 3 shows the volume of the sediment layer in the tube versus time. Strain BPY-12 dropped within 2 minutes to 8 ml, while the other three strains settle gradually to a volume of about 18 ml. These settling velocity experiments indicated that strain BPY-12 seemed to settle more quickly, although all of the strains showed strong flocculent behavior. It was noted previously however, that strain BPY-12 did not flocculate at all, until stirred. Strain BPY-11 seemed to show perhaps the second best flocculation performance.

Fermentation Characteristics

The fermentation performance of the various strains was next compared by running repeated batch fermentations with the 4 strains. The cells were initially grown anaerobically in a 5% glucose YMP media. After 24 hours, the stirring was stopped, the cells allowed to settle, the clear supernate broth removed, and a 22% glucose YMP media added to the cell sediment in the bottom of the reactor. The flasks were incubated at 32 C. and stirred with a magnetic stirrer at 200 RPM. This was done repeatedly with each of the four strains. Glucose and ethanol concentrations as a function of time for the 8th consecutive repetition are shown in Figures 4 and 5. Again we see fairly similar behavior by the various strains, with strain BPY-12 lagging in fermentation speed slightly. Each of the strains except BPY-12 were able to completely utilize all the glucose within 24 hours. Strain BPY-11 reached the highest ethanol concentration of 93 g/l. The cell concentration in the media was about 25 g/l for each of the strains.

Based on these results, we felt that strain BPY-12 was the most flocculent, but BPY-11 was also good, as well as being perhaps a slightly better or more efficient ethanol producing organisms. We therefore selected strain BPY-11 for further investigation.

Effect of reactor fluid turbulence on flocculation performance

A short experiment was performed to determine the effect of the reactor fluid dynamics on the flocculation/fermentation with strain BPY-11. A 2-liter Brunswick stirred fermenter was charged with growth media (30 g/l sucrose, YMP nutrients), and a cell crop grown for 17 hours. An air rate of 2 vvm (4 liters/min) was maintained during the aerobic experiments and a feed rate of 20 ml per hour (10 hour residence time). The reactor was run aerobically for 60 hours after which the medium was changed to 200 g/l glucose, and run anaerobically. The Reynolds number for a stirred reactor is defined as:

$$Re = D^2 N \rho / \mu$$

where D is the paddle diameter, N is the revolutions/sec, and ρ and μ are the density and viscosity of the fluid in the vessel. A Reynolds number of over 10,000 generally is required to attain turbulence throughout the reactor (Geankoplis, 1980). Our 2 liter fermenter had a 6 bladed paddle with a diameter of 3.5 cm, and one or three paddles was used depending on the desired Reynolds number.

Aerobic operations

At a Reynolds number of 1000 (stirring speed of 26 RPM) all the cells formed a mass on the bottom of the reactor. These cells could be suspended by increasing the stirring speed. After such a resuspension, the cells settled within 30 seconds to the bottom of the fermenter. The cell density in the fermenter was about 1.6 g/l with about 160 ml of cells settled on the bottom of the fermenter. The brix of the feed dropped only about 8 g/l (26% sugar utilization).

After 17 hours, the speed of the paddles was increase to give a 10,000 Reynolds number. At a reactor Reynolds number of 10,000 most of the cells remained on the bottom of the reactor. The settled cell volume was estimated at 100 ml with a density of 78 g/l. The cell density of the reactor broth was 1.4 g/l. The largest suspended cell clump had a diameter of about 0.1 mm. After a brief (1 minute) high speed agitation to lift the cells from the bottom of the reactor, the cells settled within 47 seconds, with the cell density in the supernate broth measured at 0.5 g/l.

The Reynolds number was then increased to 50,000 (1,300 RPM). At this speed, all the cells/clumps were suspended in a fairly homogenous fashion, with only a few clumps drifting on the bottom of the reactor. A settling time of 30 seconds was measured after the stirrer was stopped. A sediment layer of about 80 ml. was measured, with a cell concentration in the supernate was measured at 0.2 g/l after 30 seconds, and 0.1 g/l after 2 minutes. This Reynolds number seemed to be the minimum required to maintain the cells in suspension.

The effects of higher levels of turbulence were next tested at a Reynolds number of 180,000, (RPM of 1,500 with three paddles) which is close to the maximum we are able to attain in our lab reactor. We noted that at this velocity, the cell clumps or flakes were actually being knocked apart, with settling velocity reduced and more cells remaining in the supernate layer. After 3 hours of operation, the settling time was noted to have increased to about 2 minutes (see Figure 6) while the concentration of the cells in the broth supernate had increased from the 0.1-0.2 concentration noted earlier, to 3-5 g/l as shown in Figure 6.

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The reactor was left at this Reynolds number overnight, and the following morning (after 18 hours) it was noted that no flocs remained, with virtually no

cells settling after the stirrer was shut off. The Reynolds was then reduced to 50,000, and the reactor allows to run at a residence time of 8-10 hours. Over a period of the next 72 hours, the cells never re-flocculated. There was virtually no cells or clumps settling when the stirrer was stopped.

The reactor was then re-seeded and run anaerobically to determine if the aerobic results were representative of anaerobic fermentation. (Aerobic operation moves the fermentation toward yeast production, while anaerobic operation causes the sugars to be converted largely to ethanol). At a feed rate of 200 ml/hr (10 hour residence time) and a Reynolds number of 7,700, the cells were largely suspended. The brix in the reactor was reduced to 16 from the 20% feed. The cells maintained a small flake size of about 0.5 mm. After 16 hours, the stirring speed was increased to 300 RPM to generate a Reynolds number of 11,500. This more completely suspended all the floccs, and the brix was found to drop slightly to 15 over the next 24 hours.

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Acknowledgements

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Concentration (g/l)

1. Comparison of Cell Concentration in Down Layer

Concentration (g/l)

2. Comparison of Cell Concentration in up Layer

Sediment Layer of 50 ml Ferment Solution

3. Comparison of volume of Down Layer

Concentration (g/l)

4. Comparison of Fermentation with Four Strains

Appendix. C.

Conv. of Raw Starch to Ethanol
Dale & Lei (research rpt)

Feasibility and Availability Study for the Conversion of Raw Corn Starch to Ethanol in the Continuous Reactor Separation

M. Clark Dale & Shuiwang Lei

ABSTRACT

A system, comprised of a continuous reactor separator (CRS) column continuously producing ethanol and an absorbing column continuously removing and concentrating ethanol produced in the reactor, was developed. The CRS was a four stage reactor and total volume was about 4 liters. Four level of total solids, 10%, 15%, 20% and 30%, was used in this study. 92% of the total solids was raw corn starch and 8% was ground malt. The yeast strain used was *Kluyveromyces fragili* 2415 and the enzyme was Fermenzyme L-200 in this study. Operation temperature 38°C was selected. The experiment ran continuously for 37 days. The experiment results indicated that 79% raw corn starch was converted to ethanol in CRS. Effect of PH, yeast density and malt content on conversion of raw corn starch to glucose and glucose to ethanol was also investigated. The studies proofed that PH, Yeast density and malt ratio influenced significantly conversion of raw corn starch to ethanol.

Key wards: Ethanol processing Starch fermentation Yeast

INTRODUCTION

Ethanol can be used for motor fuel, or as a fuel additive to replace lead in gasoline, or as a chemical feedstock. Ethanol production by fermentation processing constitutes a large section of biological and agricultural engineering. More attention has been paid to fuel ethanol production from biomass since 1970. It was stimulated not only by the limitation of non-renewable petroleum source, but also by agriculture and the environment.

Corn starch is widely used as raw materials in ethanol production. Theoretical calculations indicate that processing one ton of corn could obtain 400 liters ethanol (Rhonda Hosein & Winston A. Mellowes 1988). However current batch fermentation technology for ethanol production involves liquefaction, saccharification and fermentation. These processes require large scale operation, large capital investment, and large energy consumption. About half of this energy is associated with drying and evaporating the stillage, and half with fermentation and distillation (M. Clark Dale, 1993). The Continuous Reactor Separator (CRS) is a novel system. In this system, continuous saccharification and fermentation of polysaccharides, such as starch, cellulose and other biomass,

can be quickly completed. Raw corn starch does not need to cook. Anhydrous ethanol may be obtained from feed concentration as low as 3 to 4% sugar with low energy consumption for ethanol recovery and dehydration when the CRS is coupled with solvent absorption recovery of the ethanol. This system will allow lower capital costs, lower energy usage, and lower labor costs.

Many factors are involved in the CRS. Any one of the factors will influence the conversion of raw starch to ethanol. This study focuses on the feasibility and availability for conversion of raw corn starch to ethanol in a laboratory scale CRS. Goals of the research were to determine the factor of influence on the process and to analyze the effect of the factors on conversion.

MATERIALS AND METHODS

CRS fermentation equipment. A CRS experimental apparatus was designed and built in the laboratory. This apparatus consisted of a CRS column and an absorbing column (Fig. 1). The CRS column had four stages. The volume of each stage was about one liter. The first stage volume was a little bit bigger than the others. Feed solution, enzyme, yeast, and nutrient medium were continuously fed to the top of the column. Ethanol produced in each stages was stripped with air and the stripping stream was absorbed by an absorb column. Stripping air was supplied by a compressor and absorbing water was supplied by a special pump and tank.

Feed solution. Feed mash was made of raw corn starch and ground malt. Total solids levels used in this study were 10 %, 15%, 20% and 30% by weight. The starch was 92% and malt was 8% by weight in the total solids. The starch used in this study was partly from Iowa (General Mills) and partly from market (ARGO, CPC International). The raw starch samples were dried in an oven to determine moisture content. Drying temperature was 103 °C and was for 72 hours. Average moisture content of the starch was 12.6% (wet basis). The malt came from Sigma Chemical CO. Before mixing, The ground malt was passed through a sieve NO.30 to remove bigger particles (USA Standard Testing Sieve). In order to prevent bacterial contamination, 250 ppm Sodium Bisulfite (SO₂) (J. T. Baker Inc.) was added in the solution. After mixing, PH was adjusted to desired value with ammonia and phosphoric acid (H₃PH₄) (Mallinckrodt).

Enzyme. Enzyme used in this study was Fermenzyme L-200 (Solvay Enzymes Inc.). The enzyme was a liquid glucoamylase derived from selected strains of Aspergillus niger var and capable of hydrolyzing both alpha -D-1, 6 glucosidic branchiopods and the predominating alpha -D-1,4-glucosidic linkages of starch. The enzyme was necessary to break down the starch into fermentable sugars and increase alcohol yield.

Figure 1. The CRS concept with simultaneous starch hydrolysis in a 'no-cook' process.

Microorganism. The microorganism used in this study was Kluyveromyces fragilis 2415. The strain had a high temperature tolerance and high fermentation rate. Both high temperature tolerance and high fermentation rate were desirable in fermentation and separation of ethanol production. The yeast was cultured for 24 hours before using.

Nutrient media. Two nutrient media were used in this study. The composition of the first medium was yeast extract 3 grams per liter, malt extract 3 grams per liter, and peptone 3 grams per liter. The second medium was composed of yeast extract 6 grams per liter, malt extract 6 grams per liter, and peptone 6 grams per liter. Before using, the media was autoclaved for 2 hours at temperature 120 °C.

Fermentation procedure. Continuous fermentation separation runs were performed. The feed solution, enzyme, and yeast were fed by a combined pump (Masterflex, Cole-parmert Instrument Co.). The pump was controlled by a timer. Supplying schedule of the feed was 1 minuter on and 9 minute off. The air and water was supplied continuously. Running each 24 hours, two ml sample from each stage and from the absorbing column outlet was taken. Concentrations of ethanol and glucose of the fermentation and separation were determined by high-pressure liquid chromatography (HPLC). The results were compared with a known standard ethanol and glucose concentration samples. Yeast density and remaining starch particles were checked by microscope. PH of slurry was checked by PH meter. Operating temperature was kept at 38°C. The fermentation separation experiment ran continuously for 37 days.

RESULTS AND DISCUSSION

Theoretical conversion of raw starch to sugar and sugar to ethanol. Starch is a condensed polymer of glucose. In CRS, starch was continuously converted to glucose by the enzyme and glucose was converted to ethanol by the yeast. A summery of the chemical reaction involved in this conversion is as follows:

Preliminary calculations indicate that one unit of starch could convert to 0.51 units of ethanol theoretically. However not all of starch can convert to ethanol. In the fermenting process with yeast, about 5% of the sugar is used by the yeast to produce biomass and by-products. Therefore the best result of conversion of one unit starch to ethanol was about 0.46 unit. The maximum conversion to ethanol that could be obtained in the CRS was equal to

$$\text{Ethanol (g/hr)} = 0.46(\text{g}) * \text{Feed solution (ml/hr)} * \text{Total solids (\%)} * \text{Dry matter (\%)}$$

Suppose the total solids of the feed was 20% (w/v). Starch of the total solids was 92% and the ground malt was 8% by weight. Assume malt used in this study contains 70% starch and the moisture content was as same as corn starch. Ethanol should be produced at a rate of 7.9 g per hour. The one of the results was shown in Fig. 2 and the figure was presented in Table 1. HPLC analysis was shown in Fig 3. Conversion of total solids, ethanol and glucose was indicated in Fig. 4.

Fig. 2 Conversion of Starch to Ethanol

Fig. 3 HPLC Analysis Results

Table 1 Conversion of starch to ethanol

Stage	Ethanol(g/l-hr)	Glucose(g/l-hr)
1	17.8	22
2	31.1	2.8
3	33.3	2.1
4	26.7	0.1
absorber	8.9	

The results indicate that 79% of the raw starch had been converted to ethanol. The ethanol stripped and absorbed was 57% and that remain in the last stage was 43%. The glucose was nearly exhausted by yeast. Experimental conditions were PH = 3.5 - 4.0, YMP 3,3,3. The enzyme level was 200 and yeast density was over 15 million per cubic millimeter.

Effect of malt on conversion of starch to glucose

The effect of malt on conversion of raw starch to glucose was investigated. experimental results was shown in Fig. 5 and the data was presented in Table 2.

Table 2 Effect of malt on conversion of starch to glucose

Stage	malt in total solids	
	8%	0
1	80.62	17.42
2	70.6	18.2
3	63.6	21.2
4	65.0	22.0

It is obvious that malt can help the conversion of starch to glucose. The glucose concentration using ground malt in the different stages increased to as high as about 1.9 - 3.6 times when compared to experiment using no malt. Furthermore, the saccharification time was shortened. It was helpful to accelerate conversion of glucose to ethanol.

Effect of PH on conversion of starch to ethanol

The fermentation environment was important. One of the important factor was PH. The effect of PH on conversion of starch to ethanol were studied. Test results was shown in Fig. 6A and Fig. 6B and the Data was indicated in Table 3.

Table 3 Effect of Ph on conversion of starch to ethanol

Stage	Low PH			High PH		
	Ph	ethanol	glucose	Ph	ethanol	glucose
1	2.9	0	50	3.9	12	11.4
2	2.83	4	48.6	4.04	24	1.6
3	2.88	10	41.7	4.09	25	1.4
4	2.9	16	27.8	3.98	23	1
absorber					6	

The experiments indicated clearly that yeast did not work well when PH was lowed to 3. It was much better to control the PH at about 4.

The effect of yeast density on conversion of starch to ethanol

Test results using different yeast densities are shown in Fig. 7A and Fig. 7B and the data was represented in Table 4.

Fig. 7A Effect of Yeast Density on conversion of Starch to Ethanol
Low Yeast Density

CONCLUSIONS

The CRS is a economical process in the conversion of raw starch to ethanol. It was found that 79% of raw starch was converted to ethanol on a laboratory scale.

PH and microorganism density have a significant upon the conversion of raw starch to ethanol. PH should be controlled at about 4 and yeast density should be over 15 million per cubic cent meter. Malt concentration also has an effect upon the conversion of raw starch to glucose.

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Appendix D.

The XDP for Biomass Conv. to Eth.
Dale et al. (1995)

The Xylan Delignification Process for Biomass Conversion to Ethanol

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Paper # 110 17th Annual Biotechnology for Fuels and Chemicals Symposium, Vail CO May, 1995.

Summary

An extrusion process melded with alkaline peroxide chemical pretreatments allows the lignin and hemicellulose in biomass to be solubilized, and the cellulose component to be made available for enzymatic breakdown. This process is called the Xylan Delignification Process (XDP). In this paper, some results of the XDP on promoting enzymatic breakdown and SSF of corn stalks, switch grass and straw are reported. It was found that the XDP process allowed quick (6 hour) and reasonably complete (85-88%) hydrolysis of the cellulose fraction of cornstalks, but was less effective in allowing utilization of the switch grass with 76% yield noted in 24 hours. Solubilization of the lignin and hemicellulose were not achieved on a first set of corn stalk, switch grass, and straw samples, but was noted on a second straw sample.

Key Words: Biomass Pretreatment, Ethanol, Saccharification, SSF, Delignification

Introduction

Biomass is a mix of three basic components, lignin, cellulose and hemicellulose. Lignin serves as a sort of 'glue' giving the biomass fibers its structural strength, while hemicellulose and cellulose polymers are the basic building blocks of the fibers. In order to break down the hemicellulose and cellulose to sugars, the basic structure of the biomass must be attacked. Once the structure of the biomass is disrupted, the hemicellulose and cellulose can be converted to sugars enzymatically. The conversion of biomass (through enzymatic techniques) to ethanol consists of four basic steps, 1- pretreatment of the biomass to allow enzymatic attack of the bio-polymers, 2- conversion and fermentation of the hemicellulose/xylose fraction, 3- conversion and fermentation of the cellulose/glucose fraction, and 4- separation and concentration of the ethanol. There are many different technologies currently under development to convert biomass to ethanol, but despite some rather expensive development efforts, there are no successfully operating biomass to ethanol plants running presently in the US to our knowledge.

Pre-treatment

In this work we utilized the peroxide extrusion process of Xylan. The Xylan-Delignification-Process (XDP) utilizes extrusion technology in conjunction with alkali soak and hydrogen peroxide injection. Carr and Doane (1) reported on the use of a similar process for straw pretreatment using NaOH and anthraquinone, and Chen and Wayman (2) also utilized this sort of treatment for Aspen wood chips adding SO₂ prior to extrusion with a 4% hot NaOH soak post-extrusion delignification. The Xylan method continuously treats lignocellulosic biomass by first reacting the biomass with a medium containing an aqueous solution of alkali agent (pH 11.5) which softens the lignin and allows water to enter the biomass. The cellulosic biomass is then fed into a pressurized extruder/ reactor in an oxygen atmosphere at a temperature between 235-275 F. and pressures up to 400 psi. These high temperatures and pressures allow minimization of chemicals as compared to other technologies for cellulose pretreatment (low acid-high temp, or high acid-low temp). The mechanical extrusion system mixes, grinds, sterilizes, and disrupts the cellulosic biomass cell walls. Exiting the reactor barrel is a liquid/solid mixture stream containing lignin and hemicellulose sugars, and cellulose fibers, suitable for paper, cattle feed, or enzymatic hydrolysis to glucose.

Figure 1. Xylan Delignification Process

Hydrogen peroxide is added to the barrel of the extruder to help catalyze the breakdown of the fibrous biomass structure. The wet /fibrous product from the extrusion process consists of a lignin /soluble hemicellulose stream and a fibrous cellulose. Squeezing and washing the cellulose gives two streams, the lignin/soluble hemicellulose, and the solid fibrous cellulose. The cellulose stream can be used for ethanol production, paper production, or cattle feed.

The liquid lignin/soluble hemicellulose stream is easily converted to xylose enzymatically, after which it can be converted to ethanol using various fermentation strategies. In previous lab tests, when treating this stream (from a pre-treated wood chip process) with hemicellulase, good conversion of the hemicellulose to xylose was noted, with 20 g/l xylose being determined after hydrolysis along with 2.2 g/l of glucose (3). The cellulosic fibers stream can then be broken down to glucose via a similar enzymatic treatment. The cellulosic fiber stream, when added to water and hydrolyzed with cellulase gave a 10.7 g/l ethanol product during a Simultaneous Saccharification Fermentation (SSF) with a 31.9% yield of ethanol from dry matter yield (3). Co-products of the fermentation included lactic acid and acetic acid at 3.3 and 1.6 g/l final concentrations respectively with 4.2 g/l glucose also remaining unfermented.

Methods

This project was funded largely by NREL, who have established a set of standard procedures to allow work by various contractors to be directly comparable (4). These procedures were then used for determinations of biomass compositions and fermentation broths as described below.

Ethanol- 0.1 ml. sample is diluted with 0.9 ml of solution containing isopropyl alcohol (internal standard) and analyzed using a gas chromatograph fitted with a carbowax column.

Total solids of biomass- The sample (1-5 g) is held at 105 C for between 3 to 24 hours - until constant weight is attained.

Klason Lignin- The sample is digested in 72% sulfuric acid, with the lignin remaining undigested and determined by gravimetric analysis. Three samples were run to establish the repeatability of the analysis.

Acid soluble lignin-Acid soluble lignin is determined by spectrophotometric absorbance at 205 nm on the filtrate.

Ash- The sample is ashed in a furnace at 575 C for 3-5 hours. There may be a slight increase in ash due to the chemicals added in the XDP processing.

Cellulase Activities- Cellulase, Lot # 17-92262-09 from Env. Biotech. Inc. was utilized to release sugars from the biomass. The FPU (enzyme quantity able to breakdown 4% of a filter paper solution) of the cellulase esymes provided was determined as 67.9 FPU/ml av. dev +/- 0.76 FPU/ml with three determinations run.

Saccharification of biomass samples- The hydrolysis of the biomass with the cellulase was measured by taking 0.1 g of cellulose in the biomass sample (by calculation based on dry weight and cellulose composition) added to 9.9 ml water. 66.7 microliters of cellulase enzyme is then added to the solution, and the sample is then allowed to digest at 50°C. Glucose liberation rate is then measured.

SSF of XDP samples- Approximately 30 g/L of cellulose (based on the cellulose content of the biomass analysis) was simultaneously saccharified and fermented with an enzyme dosage of 25 FPU/ml in the fermentation mixture (actual biomass dosages are given in Table 1). Yeast *S. cerevisae* D5A was used unless noted otherwise with the fermentation carried out at 38°C. The biomass stream was fermented with three fermentations carried out simultaneously. An alpha-cellulose sample was fermented as a baseline control sample. Table 1 gives the amount of biomass (wet basis) added to the various saccharification and fermentation trials with total fermentation broth being 50 ml.

Table 1.

Procedure(4)	Figure	Substrate	g Biomass (wet wt)
008-Hydr	1	XDP corn stalks	2.6g
008-Hydr	2	XDP switch grass	2.7g
008-Hydr	3	fresh corn stalk	2.0g
008-SSF	4	XDP corn stalk	7.0g
008-SSF	5	XDP switch grass	4.5g
008-SSF	7	XDP corn stalk	5.0g

Pretreatment- The process begins with the addition of sodium hydroxide, at approximately 3# of 50% NaOH solution per 100 # of biomass, The base was mixed with the shredded biomass in a 15HP Grainger stainless steel mixer for a period of fifteen minutes. The biomass was then taken to a "cram feeder" attachment to a SX 20 Wenger extruder. The barrel of the extruder was heated to 200 °C using low pressure steam. Hydrogen peroxide, at about a 9% solution strength, was injected near the entrance to the extruder barrel at a rate of about 1 # per 100# of biomass. A pressure of about 300 to 400 psi

develops within the extruder barrel. After about a 15 second residence time, the biomass 'exploded' from the die at the end of the extruder barrel. A 3/8" die aperture was used when processing the straw, corn stalks, and switch grass used in this project.

Lab Analysis

The XDP treated samples of straw, corn stalks and switch grass were received at Purdue from Mr. Tyson of Xylan, Inc. Biomass samples were sealed in zip-lock bags and frozen to prevent deterioration. The samples were tested for basic composition as given below:

	Corn Stalk	Switch Grass	Straw
1. Total Solids	38.0%	56.8%	87.9%
av. dev.	+/- 0.47	0.85	0.3%
2. Ash	15.0%	11.4%	10.7%
3. K. Lignin	18.8%	21.0%	23.4%
4. A. S. Lignin	2.0%	1.7%	0.3%
5. Acid Hydrol.			
a) Glucose	39.2%	31.4%	39.5%
av. dev.	+/-0.34	1.1	1.0%
b) Xylose	24.9%	20.5%	19.1%
av.dev.	+/-0.3	2.6	0.3%

Results

Enzymatic Hydrolysis of XDP Samples The results of enzyme hydrolysis (w/ fermentation nutrients) of XDP treated corn stalks are shown in Figure 1. The yield or conversion efficiency (as defined as grams cellobiose and glucose released per gram of cellulose) can be seen to be about 85% within 7 hours. During hours 7 through 24, cellobiose concentration drops with a concomitant increase in glucose concentration. Final glucose concentrations of 4.8 g/l glucose, 3.2 g/l cellobiose, and 2.6 g/l xylose were noted. The variability between the three replicates is given as a range about the average.

Similar data for XDP treated switch grass is shown in Figure 2. Conversion efficiencies of 78% were noted at 24 hours, with conversion being somewhat slower than was seen with the XDP treated corn stalks, although a 65% conversion yield was noted at 7 hours. Cellobiose levels were not observed to drop, although glucose levels continued to rise slowly from hours 7 to 24 of the treatment. As a control, alpha-cellulose showed a 97% conversion to cellobiose and glucose in a 24 hour period.

The effectiveness of the XDP treatment as compared to untreated corn stalks was determined finally as a test of the XDP efficacy. 'Fresh' corn stalks were gathered from an Indiana field about 2 months after harvest (late October '94), manually cut into short 2 cm lengths, milled in Waring blender, and sieved using a 40 mesh sieve. This untreated corn stalk sample was then subjected to the same enzymatic evaluation as the pretreated samples. As shown in Figure 3, conversion was considerably slower, with only a 50% yield noted in 7 hours, and a final yield of 72% noted at 24 hours.

It was determined that the yeast nutrients had a beneficial effect or possibly some cellulosic enzymatic properties when the hydrolysis experiments were repeated without the fermentation nutrients on the XDP corn stalks, the XDP switch grass, and the alpha cellulose. XDP treated corn stalks showed a much slower release of sugars, with a 65% yield determined at 95 hours, and a final yield of 75% determined at 170 hours. Switch grass showed similar drop in performance without the fermentation nutrients a 55% yield was determined at 50 hours, with a final yield of 72% noted at 170 hours (5). Alpha cellulose as well was found to be much slower to convert with a 98% conversion recorded at 160 hours as compared to 24 hours when nutrients were present.

SSF of XDP treated Biomass The Simultaneous Saccharification and Fermentation (SSF) of the biomass samples was next tested with the D5A *S. cerevisiae* as provided by NREL, and then repeated using some of our labs yeast cultures. The SSF of XDP treated corn stalks is shown in Figure 4. We measured a 46% yield in 48 hours with an ethanol concentration of 12 g/liter, after which time there was little change in ethanol level over the next 75 hours. SSF of the XDP treated switch grass is shown in Figure 5 with a 33% yield noted at 48 hours, after which time ethanol levels were observed to drop with time. An ethanol concentration of close to 6 g/l was reached at 48 hours. SSF of alpha cellulose was run as a control with 16.5 g/l ethanol reached at 100 hours. Yields from the various biomass samples are compared in Figure 6, corn stalks and alpha cellulose fermentation rates were comparable over the first 50 hours, after which alpha cellulose continued to convert, while the corn stalk fermentation was stagnant. Switch grass performance was significantly lower than corn stalks.

SSF of the XDP treated cornstalks was run using a flocculent *S. cerevisiae* maintained in our labs, strain NRRL 11878. This yeast is not as temperature tolerant as the D5A strain, so the fermentation was run at 30°C. Performance was much worse at this lower temperature, with only a 25% yield noted at 48 hours. A residual glucose

concentration of 2 g/l was noted with no measurable xylose observed. Co-culturing of glucose fermenting (*S. cerevisiae* 11878) and xylose fermenting (*P. stipitus* NRRL 11545) yeast strains was next tested (similar to studies by Grooten et al, (6,7)). Yield improved slightly to 33% in about 48 hours, with lower levels of glucose determined (0.5 g/l) at 70 hours. Again, no xylose was noted in our analyses.

A comparison of the SSF experiments on XDP corn stalks is shown in Figure 7. The higher temperature SSF gave the best yields. The use of even higher temperature yeast, perhaps *K marxianus* strains might be explored in the future (8). In results not shown here, it was found that contamination of the SSF broth led to poor fermentation performance. We found it important to filter sterilize the cellulase enzyme to prevent any contamination of the SSF experiments.

Solubilization of lignin and hemicellulose by the XDP The XDP treatment of biomass should have the effect of solubilizing the lignin and hemicellulose fractions of the biomass. The effectiveness of the XDP treatment was evaluated by a few tests in which the biomass was rinsed with hot water to release the soluble hemicellulose and lignin. If the XDP were totally effective, little lignin or hemicellulose would remain after the rinsing, with the fiber being largely cellulose. Acid hydrolysis of the rinsed remaining cellulose fiber should release only glucose. Solubilization tests of four substrates are shown in Table 2. As per this table, only 12.2-15.6% of the solids obtained during '94 were soluble, but the second straw sample received in early 1995, which had been run through a different extruder, showed good solubility (Straw II).

Table 2. Solubilization of Biomass by the XDP

	Corn Stalks	Switch Grass	Wheat Straw	Straw II
Dry Solid/50 g	20.5	28.3	44.1	44.9
Soluble Solids	2.52	4.4	6.6	14.5
% Soluble Solids	12.20%	15.60%	14.90%	32.30%

The enzymatic hydrolysis of this second sample of straw (Straw II) was tested. The product was separated into two streams as per Figure 1, a solids fraction remaining after water extraction, and the liquid stream containing the solubles. The liquid stream was treated with hemicellulase as shown in Figure 8, with an initial xylose concentration of 5.3 g/l increasing to a final concentration of 6.8 g/l in 3 hours. Similarly, the solid stream was hydrolyzed as shown in Figure 9. We measured a 68% yield of the cellulose to sugars

(yield defined based on compositions shown in Table 1) in 12 hours with a fairly large amount of xylose also released (an initial concentration of 2 g/l increasing to 8.0 g/l over 12 hours) indicating that the recovery of hemicellulose/xylose in the liquid extraction was not total. A mass balance on the xylose indicates 48% of the hemicellulose was extracted in the hot water extraction, perhaps more could have been extracted in repeated washings. These results make us suspect that the correct extrusion temperatures/pressures were not quite reached during the pretreatment process in the original 3 samples, but was achieved in the second straw sample (Straw II).

Discussion

We feel that the XDP for biomass pretreatment could be coupled with a two stage fermentation process to produce ethanol from biomass in an efficient and economical fashion. The solubilized liquid stream could be fermented using an efficient xylose to ethanol yeast, while the cellulose could be fermented using either a thermotolerant glucose to ethanol yeast/bacteria in an SSF type reactor, or pre-hydrolysed and fermented. Co-fermentation of xylose and glucose is difficult as noted by Grooten et al. (6,7)), although apparently some progress is being made on the development of a genetically altered yeast which can sequentially ferment both xylose and glucose (9). To achieve a high concentration of xylose, a counter current washing of the biomass could be used. In a preliminary simulation of this process, we found that when a solution was used 4 times to extract solubles from four straw II samples, acetic acid built up in an almost linear fashion from 1.2 g/l in the first rinse to 7.6 g/l in the fourth rinse. A recent study (10) suggests that the acetic acid is quite inhibitory to *P. stipitus*, with no fermentation noted at 5 g/l acetic acid and little at 2 g/l. The removal of the acetic acid was required to achieve a fast and efficient conversion of the hemicellulose hydrolysis.

Acknowledgments

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Figure 1. Hydrolysis of XDP-treated Corn Stalk

Figure 2. Hydrolysis of Switch Grass

Figure 3. Hydrolysis of Non-treated Corn stalk

Figure 4. SSF of XDP-treated Corn Stalk

Figure 5. SSF of XDP-treated Switch Grass

Figure 6. Comparison of Biomass SSF

Figure 7. Comparison of Biomass SSF
(Different Strains and Temperature)

Figure 8. Hydrolysis of Extracted Liquid
(XDP-treated Wheat Straw II)

Figure 9. Hydrolysis of Extracted Wheat Straw Solids
(XDP-treated Wheat Straw II)

Appendix. E

Xylose & Xylose/Glucose Ferm. by Yeast
Zhao & Dale (research rpt)

Xylose and Xylose/Glucose Fermentation by Yeast: A Strain Comparison

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Abstract

The ability of 5 strains of xylose fermenting yeast to convert xylose and xylose/glucose mixtures to ethanol were compared. *Pichia stipitis* NRRL 7124, *Pichia stipitis* NRRL 11545, *Pachysolen tannophilus* NRRL 2460, and *Pichia segobiensis* NRRL 11571 and Tygel 12 from Xylan, Inc. It was found that *Pichia stipitis* 11545 was the best performing xylose/glucose to ethanol strain in these experiments. *Pichia stipitis* 7124 and 11545 performed similarly on 4 and 6% xylose fermentations with both strains able to produce about 20 g/l ethanol on a pure xylose feed, but strain 11545 showed substantially better performance on mixtures of glucose and xylose. Strain 11545 was able to produce up to 27 g/l ethanol on mixtures of glucose and ethanol. Xylose was not utilized in glucose/xylose mixtures until the glucose level was less than 15 g/l. Yeast extract was shown to speed the fermentation as compared to corn steep. *Pichia segobiensis* 11571 showed stronger ethanol inhibition than the two *Pichia stipitis* strains, with fermentation ceasing at 10 to 11 g/l., while Tygel 12 showed good growth on xylose, but no measurable ethanol production in semi-anaerobic fermentation mode was noted.

Introduction

There is a great deal of interest in converting wood and biomass streams to renewable fuels. The basic composition of these ligno-cellulosic materials is a mix of three major components, cellulose which breaks down to glucose, hemicellulose which breaks down primarily to xylose, and lignin. The economics of biomass conversion to ethanol are improved if both the hemi-cellulose and the cellulose are converted to ethanol (Ladisich and Swartzkopf, 1993). The conversion of xylose to ethanol is being intensively studied with a variety of strains found to be able to efficiently convert xylose to ethanol. These strains include *Pichia stipitis*, *Pachysolan tannophilus*, *Candida shehatae*, and genetically modified yeast and bacteria. Thermophilic anaerobic conversion of

cellulose to ethanol by *T. cellulum* has been demonstrated by several researchers (Zertuche and Zall, 1982; Shahbazi et al, 1991) but this organism is only able to produce 5 to 7 g/l or less of ethanol, and generally produces a number of side products (organic acids). The conversion of xylose to ethanol by *Pichia stipitis* has been recently discussed by Laplace et al (1991) who compared *Pichia stipitis* 7124 and a strain of *Candida shehatae*. They found 7124 gave a better conversion efficiency ($Y_{p/s}$ of 0.44) as compared to the *Candida shehatae* (0.40) with both strains giving similar maximal ethanol concentration of about 30 g/l. Grootjen et al (1990) determined a maximal ethanol productivity of 0.13 g eth/g cell hr for *Candida shehatae* CBS 5773. They determined that there was a maximal yield ($Y_{p/s}$) of 0.31 g eth/g xylose when no oxygen was added to the fermenter, with yield then falling to zero ethanol if oxygen at 0.03 moles/l*hr was added to the fermenter (the cells going to an aerobic biomass production mode). Dominguez et al (1993) determined maximal growth rates (μ_{max}) and substrate affinity constants (K_s) from the Monod model for *Pichia stipitis* 7124 under aerobic and anaerobic environments. Values of 0.19 g/g*hr were determined for specific growth rates under aerobic conditions as compared to 0.066 g/g*hr under anaerobic conditions. Guebel et al (1991) also determined that growth was very slow under anaerobic conditions for *Pichia stipitis* 7124, while at 5 mmol O₂/l*hr, a maximal growth rate of 0.11 g/g*hr was noted. An ethanol yield of .34 g ethanol/g xylose was measured under anaerobic conditions, while at 5 mmol O₂/l*hr, yield fell to 0.28 g/g. The effects of combined glucose and xylose mixtures on *Pichia stipitis* behavior has been studied by Grootjen et al (1991). It was determined that with a 40 g/l glucose and 10 g/l xylose, the *Pichia stipitis* would preferentially metabolize glucose with only 20% of the xylose utilized (either for cell growth or ethanol production). A steady state ethanol concentration of 14 g/l was attained in a CSTR. It was determined from this experiment that simple utilization of the glucose by a *Saccharomyces cerevisiae* yeast would give comparable or better results.

The effects of pH and temperature on *P. stipitus* 7124 growth and productivity was evaluated by Slininger et al(1990). A wide optimum for pH between 4 and 7 and for temperature of 25 to 33 for cell growth was noted, with a μ_{max} of 0.55 g/g h. Similarly, for productivity, a v_{max} of 0.16 and P_m of 45-50 (30°C) to 55 (25°C) was noted between pH 4 and 6. Maximum yields ($Y_{p/s}$) of about 0.37 at 25°C can be determined from data presented. Growth rates and ethanol tolerances were higher in this study than in other reports, perhaps due to improved oxygen transfer rates.

Genetically engineered *Saccharomyces cerevisiae* able to ferment xylose have been reported by Tantirungkij et al (1993) who were not able to actually generate much ethanol, and Ho et al (1994) who seems to have successfully completed a fermentation of both glucose and xylose with the transformed yeast. A recombinant *E. coli* has been shown to be able to ferment xylose well, with optimal pH of 6 to 7 (Bealle et al, 1991). Actual fermentation of biomass hydro-

lysate has not been able to duplicate these results, with ongoing experiments trying to determine the components poisoning the fermentation (Bothast, 1994).

The basic purpose of this study was to compare some of the better strains of xylose to ethanol yeast.. We are investigating a process in which hemicellulose/lignin are first separated and hydrolysed based on the Xylan process (Tyson et al, 1993). In this process biomass is mechanically extruded at high temperatures and pressures in the presence of some chemicals. Carr and Doane (1984) reported on the use of a similar process for straw pretreatment. This Xylan Delignification Process (XDP) causes a breakdown of the lignin and hemicellulose which can then be separated from the cellulose by rinsing. Fermentation of the xylose can then occur prior to cellulose fermentation. This pre-saccharification of xylose eliminates the problem of trying to ferment xylose in the presence of glucose. Gorton et al (1991) suggests a similar strategy following the poor results noted in his co-fermentation experiments.

Methods

Four strains of xylose fermenting yeasts were obtained from C. Kurtzman of the NCAUR (*Pichia stipitis* NRRL 7124, *Pichia stipitis* NRRL 11545, *Pachysolen tannophilus* NRRL 2460 and *Pichia segobiensis* NRRL 11571), and one strain from Xylan Inc. (Tygel 12). Each of these strain was grown up aerobically (1 vvm air through an airstone sparger, 300 ml broth in a 500 ml erlenmeyer flask) at 25 C. in a media consisting of 20 g/l xylose and 20 g/l corn steep liquor. The final cell densities ranged from 9.5 to 11 g/l dry basis. These cells were separated from the cell broth and added to the test fermentation media at a cell concentration of 5 to 7 g/l cells. Test fermentations of 60 ml were performed in 150 ml flasks held in an incubator at 30 C. Each flask was stirred at 180 RPM using a magnetic stirrer/stir bar. The flasks were capped with a foam plug covered with foil. A sulfite oxidation test (ref) indicated an oxygen transfer rate of 8.75 mmol/l h under these conditions.

The standard test medium consisted of the test sugar(s) and a nutritional supplement of 8 g/l yeast extract. Some of the latter experiments tested the effects of different nutritional supplements with the effects of yeast extract compared to corn steep liquor. Ethanol, xylose, and co-product changes in concentration were monitored by analysing samples from the batch reactions in a HPLC fitting with an HPX87H aminex column (Biorad) and a refractive index detector.

Results

The basic fermentation performance of *Pichia stipitis* 11545, *Pichia stipitis* 7124, *Pichia segobiensis* 11571 and *Pachysolen tannophilus* 2460 on the fermentation of a 35 to 40 g/l xylose medium are shown in Figures 1 through 4. It can be seen that *Pichia stipitis* 11545 and 7124 fermented the xylose quite

effectively. An ethanol yield ($Y_{p/s}$, gm ethanol per gm xylose) of 0.46 and 0.36 were determined rectively. *Pachysolen tannophilus* 2460 (Figure 3), however, showed substantial quantities of xylulose co-product ($Y_{co-p/x}$ of 0.35) reducing the ethanol yield coefficient to 0.29. Fermentation of 60 g/l xylose was next examined as shown in Figures 5 through 8. *Pichia stipitis* 11545 slightly outperformed *Pichia stipitis* 7124 in this experiment, with strain 11545 dropping the sugar concentration to 21 g/l in 120 hours, while strain 7124 dropped the xylose to 25 g/l. Strain *Pichia stipitis* 11545 also gave a better yield than 7124 at this higher sugar concentration, (0.46 versus 0.43 g/g). *Pachysolen tannophilus* 2460 showed a considerably poorer performance with only 14 g/l ethanol being produced in 120 hours, while *Pichia segobiensis* 11571 showed only 10 g/l in ethanol 120 h (Figure 7 and 8). In repeated trials, the yeast Tygel 12 showed good aerobic and anaerobic growth on xylose, but no measurable ethanol was produced in any trials.

The effects of glucose and xylose in the same fermentation mixture was studied. The four strains were fermented in a fermentation broth containing 50 g/l xylose and 40 g/l glucose. Strain *Pichia stipitis* 11545 showed preferential use of the glucose followed by a slow fermentation of the xylose as shown in Figure 9. Glucose was totally utilized within 50 hours. Xylose began to be fermented once glucose levels were under 15 g/l. A final ethanol concentration of 26 g/l was reached at 120 hours, with a yield, $Y_{p/s}$, of 0.35 g/g. Strain *Pichia stipitis* 7124, suggested by Laplace et al (1991) as a top performer, showed a poorer performance (Figure 10). Glucose and xylose seem to both be metabolized, but only very slowly.. Glucose levels dropped from 43 to 14 g/l, xylose from 50 to 36 g/l, and ethanol increased from 0 to 12 g/l over 120 hours for a net yield ($Y_{p/s}$ of 0.28. *Pichia segobiensis* 11571 also first metabolized glucose, with all the glucose metabolized in 44 hours. At this point, some xylose began to be very slowly converted to ethanol. A higher ethanol level was developed in this mixed media (19 g/l) than was attained on xylose media (10-11 g/l). Strain *Pachysolen tannophilus* 2460 showed a strong preference for glucose as did *Pichia stipitis* 11545. As seen in Figure 12 glucose was utilized by 50 hours, and xylose dropped to 30 g/l by 70 hours, after which the fermentation ceased. A final ethanol concentration of 20 g/l was reached with a yield, $Y_{x/s}$ of .31 g/g. Interestingly, there was little production of xylulose during this co-sugar fermentation.

These experiments suggest that strain *Pichia stipitis* 11545 has definite performance advantages over the other 4 strains. In a pure xylose fermentation, *Pichia stipitis* 11545 and 7124 behave almost identically, while in a mixed sugar environment, *Pichia stipitis* 11545 definitely fermented more quickly than the 7124. Strain *Pachysolen tannophilus* 2460 showed a propensity towards xylulose production when fermented on pure xylose, but performed similarly to *Pichia stipitis* 11545 on mixed sugars, showing a better performance on glucose than on xylose. These trials suggest that strain 11545 is the best performing ethanol producer of the 5 strains of yeast examined for the

production of ethanol from xylose and xylose/glucose mixtures.. We therefore ran some further experiments on strain *Pichia stipitis* 11545.

The effects of various glucose/xylose levels on *Pichia stipitis* 11545 is shown in Figure 13. These experiments verified what was seen in our previous experiment (Figure 7), that xylose will be fermented when glucose levels are under 15 g/l. When glucose levels are over 15 g/l, very little xylose is fermented. High sugar levels are somewhat inhibitory, with the highest ethanol concentration (25 g/l) achieved from the fermentation of a mix of 60 g/l glucose, 30 g/l xylose (experiment 18). Xylose was only utilized in experiment 19 where initial glucose was 30 g/l, and experiment 16 with no glucose (95 g/l initial xylose). If initial fermentation rates are used to determine specific fermentation rate, n , g ethanol/ g cells per hour, a value of 0.068 can be determined from experiments at 4, 6, and 10% xylose. A similar rate was noted during the conversion of glucose in mixed fermentations (Figure 9). Ethanol inhibition seems complete at about 30 g/l, so a general model for the xylose fermentation with strain *Pichia stipitis* 11545 can be written as:

$$v = v_{\max} (1 - P/P_m)$$

where $v_{\max}$ is 0.068, and P_m is 30 g/l. These values are considerably lower than values determined by Slininger et al (1990) of $v_{\max}$ of 0.14, and P_m of 55 g/l for strain 7124. This is probably due to the addition of oxygen (K_{La} of 0.165/min) to the fermenter by this group, giving the higher rates but with a concomitant loss in yeild (0.37) due to cell growth during the fermentation.

Some nutritional experiments are shown in Figure 14, where various nutritional supplements were added to a 55 g/l xylose medium. Replacement of yeast extract with various levels of corn steep were not very successful. Very little difference in fermentation rates was noted at the three levels of corn steep added (0.33, 0.67, and 1% levels), with the lower level of corn steep actually fermenting a little bit faster than the higher levels. The addition of yeast extract (5 g/l) caused the fermentation to proceed more quickly (v of 0.045 g eth/g cell*h) than the fermentations supplemented with corn steep liquor (v of 0.023 g/g*h).

Conclusions

Strain *Pichia stipitis* 11545 was selected from among 5 strains of xylose metabolizing yeasts. This strain shows an ability to produce up to 3% ethanol from a 6% xylose or xylose/glucose mix. Specific productivity was determined to be about 0.042 g/g*h for this strain on xylose in a low oxygen transfer environment. A good conversion efficiency of 0.44 to 0.46 was shown on pure xylose substrates (85-90% of theoretical conversion). Strain *Pichia stipitis* 7124 performed similarly as strain 11545 on xylose, but showed considerable glucose inhibition in mixed sugar fermentations. Strain *Pichia segobiensis* 11571 showed

a good conversion efficiency but was more strongly ethanol inhibited with 10 to 11 g/l ethanol maximum ethanol on pure xylose. Strain *Pachysolen tannophilus* 2460 was a less effective xylose fermenter, producing approximated equal weights of xylulose and ethanol. Tygel 12 grew well on xylose, but produced no measurable amounts of ethanol.

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Figure 1. Fermentation of 4% D-Xylose with *Pichia stipitus* 11545

Figure 2. Fermentation of 4% D-Xylose with *Pichia stipitus* 7124

Figure 3. Fermentation of 4% D-Xylose with *Pachysolen tannophilus* 2460

Figure 4. Fermentation of 4% D-Xylose with *Pichia segobiensis* 11751

Concentration (g/l)

Figure 5. Fermentation of 6% D-Xylose with *Pichia stipitus* 11545

Figure 6. Fermentation of 6% D-Xylose with *Pichia stipitis* 7124

Figure 7. Fermentation of 6% D-Xylose with *Pachysolen tannophilus* 2460

Figure 8. Fermentation of 6% D-Xylose with *Pichia segobiensis* 11751

Figure 9. Fermentation of 4% Glucose/5% D-Xylose with *Pichia stipitus* 11545

Figure 10. Fermentation of 4% Glucose/5% D-Xylose with *Pichia stipitus* 7124

Figure 11. Fermentation of 4% Glucose/5% D-Xylose with *Pachysolen tamnophilus* 2460

Figure 12. Fermentation of 4% Glucose/5% D-Xylose with *Pichia segobiensis* 11751

Concentration (g/l)

Figure 13. Fermentation of Glucose, D-Xylose with *Pichia stipitius* 11545

(11545)

Concentration (g/l)

(11545)

Appendix.F.

A Pilot CSRS for Ethanol from Biomass
(condensed. ERIP follow on propos)

**A Pilot Plant Scale Reactor/Separator
for the Reduced Energy
Production of Ethanol from Cellulosic Materials.**

A Proposal to the Energy Related Inventions Program

Project Director:

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Table of Contents

	page
Executive Summary.....	3
1. Technical Description.....	4
2. Technical Advantages.....	8
3. Energy Impact.....	10
4. Cost and Economic Considerations.....	10
5. Commercial Potential and Market.....	12
Bibliography.....	19

Executive Summary

A Pilot Plant Continuous Stirred Reactor Separator for the Production of Ethanol from Cellulosic Materials is a proposal in which BPI follows up our current project with the NIST-DOE Energy Related Inventions Program (consisting of converting starch and sugars to ethanol in a Continuous Stirred Reactor Separator (CSRS) on the lab and pilot scale) with the application of the CSRS reactor technology to pretreated cellulosics. Lab work on process development for cellulose conversion is being done under the current project, and these efforts will be continued during the first 12 months of this new project, and then followed by a demonstration scale test of ethanol production from biomass/cellulosics using a 15,000 L. CSRS. This CSRS will be able to utilize the low energy SAED (solvent absorption-extractive distillation) ethanol recovery system designed and built as a part of the current project.

A high pressure-high temperature extrusion pretreatment (the Xylan Delignification Process) of biomass will be followed by a two stage enzymatic hydrolysis and fermentation process in the CSRS. A hot rinsing of the pretreated biomass will give a 4 to 6% hemicellulose /lignin stream. The first portion of the fermentation process is a simultaneous saccharification and fermentation (SSF) of this hemicellulose-xylose stream which is solubilized by the Xylan pretreatment. A strain of *Pichia stipitus* (which has been found to give very good conversion efficiencies of xylose to ethanol in our current studies) will be used to ferment the xylose as it is released. Following this simultaneous saccharification and fermentation (SSF) of hemicellulose in stages 1 and 2, the cellulose fibers are added to the broth for the second portion of the fermentation, the SSF of cellulose-glucose which occurs in stages 3, 4 and 5. These processes are combined with ethanol separation in the CSRS to allow high speed fermentation of the released xylose and glucose. Process kinetics and small scale batch and continuous tests will be performed at BPI's laboratory during the first 12 months of the project, with some tests performed on enzyme development and comparisons of commercial cellulases and hemi-cellulases at Purdue University as a sub-contract. A process model/design will be developed to allow optimization of the process conditions and to give cost estimates for biomass to ethanol facilities as a function of scale.

Total costs for the project are estimated at \$347,750 over a 24 month period. In this proposal we request \$92,500 from NIST-ERIP , with \$255,250 being provided by other project co-sponsors:.

Section 1. Technical Description

Introduction

The conversion of biomass (through enzymatic techniques) to ethanol consists of four basic steps, 1- pretreatment of the biomass to allow enzymatic attack of the bio-polymers, 2- conversion and fermentation of the hemicellulose/xylose fraction, 3- conversion and fermentation of the cellulose/glucose fraction, and 4- separation and concentration of the ethanol. There are many different technologies currently under development to convert biomass to ethanol, but despite some rather expensive development efforts, there are no successfully operating biomass to ethanol plants running presently in the US to our knowledge. In this proposal, we are focusing on an enzymatic, two stage conversion process of biomass to ethanol, using the CSRS reactor technology to speed fermentation rates. We feel as though this technology may have substantial advantages over acid based or competing enzymatic based processes which are under development by various organizations.

Biomass is a mix of three basic components, lignin, cellulose and hemicellulose. Lignin serves as a sort of 'glue' giving the biomass fibers its structural strength, while hemicellulose and cellulose polymers are the basic building blocks of the fibers. In order to break down the hemicellulose and cellulose to sugars, the basic structure of the biomass must be attacked. Once the structure of the biomass is disrupted, the hemicellulose and cellulose can be converted to sugars enzymatically.

Pre-treatment

In this project we will utilize the peroxide extrusion process and cooperate with Xylan Inc who have developed this technology. Xylan Inc has agreed to both provide samples of different cellulosic materials pretreated using their process and to provide one truck load (60,000 pounds) of pretreated corn stalks for our pilot scale test. The Xylan-Delignification-Process (XDP) utilizes extrusion technology in conjunction with alkaline hydrogen peroxide. Carr and Doane (1984) reported on the use of a similar process for straw pretreatment, and Chen and Wayman (1989) also utilize this sort of treatment for Aspen pretreatment. The Xylan method continuously treats lignocellulosic biomass by reacting the biomass with a reaction medium containing an aqueous solution of alkali agent (pH 11.5) which softens the lignin and allows water to enter the biomass. The cellulosic biomass is then fed into a pressurized extruder/ reactor in an oxygen atmosphere at a temperature between 235-275 F. and pressures up to 400 psi. These high temperatures and pressures allow minimization of chemicals as compared to other technologies for cellulose pretreatment (low acid-high temp, or high acid-low temp). The mechanical extrusion system mixes, grinds, sterilizes, and disrupts the cellulosic biomass cell walls. Exiting the reactor barrel is a liquid/solid mixture stream containing lignin and hemicellulose sugars,

and cellulose fibers, suitable for paper, cattle feed, or enzymatic hydrolysis to glucose. Exit temperatures from the extruder are in the range of 115 to 125 C.

Figure 1. Xylan Delignification Process

Tyson (1993) suggests that temperatures of 138 C and a pressure of 340 psi seemed best during tests with hardwoods. Hydrogen peroxide is added to the barrel of the extruder to help catalyze the breakdown of the fibrous biomass structure. The wet /fibrous product from the extrusion process consists of a lignin /soluble hemicellulose stream and a fibrous cellulose. Squeezing and washing the cellulose gives two streams, the lignin/soluble hemicellulose, and the solid fibrous cellulose. The cellulose stream could be used for ethanol production, paper production, or cattle feed. In this project, we will be concentrating on ethanol production from this fibrous solid material.

The liquid lignin/soluble hemicellulose stream is easily converted to xylose enzymatically, after which it can be converted to ethanol using various fermentation strategies. In previous lab tests, when treating this stream (from a pre-treated wood chip process) with hemicellulase, the conversion of the hemicellulose to xylose was noted, with 20 g/l xylose being determined after hydrolysis along with 2.2 g/l of glucose (Tyson, 1993). The cellulosic fibers stream can then be broken down to glucose via a similar enzymatic treatment. The cellulosic fiber stream, when added to water and hydrolyzed with cellulase gave a 10.7 g/l ethanol product during a Simultaneous Saccharification Fermentation (SSF) with a 31.9% yield of ethanol from dry matter yield (Tyson, 1993). Co-products of the fermentation included lactic acid and acetic acid at 3.3 and 1.6 g/l final concentrations respectively with 4.2 g/l glucose also remaining unfermented (Tyson, 1993). SSF of corn stalks pretreated by the Xylan process as run in a batch test are shown in Figure 2 (Tyson, Dale, Zhou and Lei, 1995)

Fermentation

The major goal of this project is operation of a demonstration scale CSRS unit running on biomass materials. We plan to build a 15,000 liter pilot plant to demonstrate the cellulose to ethanol technology shown in Figure 3. The economics of biomass conversion to ethanol are greatly improved if both the hemi-cellulose and the cellulose are converted to ethanol (Ladisich and Swartzkopf, 1993). This Xylan Delignification Process (XDP) causes a breakdown of the lignin and hemi-cellulose which can then be separated from the cellulose by rinsing. Fermentation of the xylose can then occur prior to cellulose fermentation. This pre-saccharification of xylose eliminates the problem of trying to ferment xylose in the presence of glucose. Grooten et al (1991) suggests a similar strategy following the poor results noted in his co-fermentation experiments. The conversion of xylose to ethanol is recently been improved with a variety of strains found to be able to efficiently convert xylose to ethanol. These strains include *Pichia stipitis*, *Pachysolen tannophilus*, *Candida shehatae*, and genetically modified yeast and bacteria. Thermophilic anaerobic conversion of cellulose to ethanol has also been demonstrated by several researchers (Zertuche and Zall, 1982; Shahbazi et al, 1991) but this organism is only able to produce 5 to 7 g/l or less of ethanol, and generally produces a number of side products (organic acids).

Genetically engineered *Saccharomyces cerevisiae* able to ferment xylose have been reported by Tantirungkij et al (1993) who were not able to actually generate much ethanol, and Ho et al (1994) who seems to have successfully completed a fermentation of both glucose and xylose with a transformed yeast. A recombinant *E. coli* has been shown to be able to ferment xylose well, with optimal pH of 6 to 7 (Bealle et al, 1991). Actual fermentation of biomass hydrolysate has not been able to duplicate these results, with ongoing experiments trying to determine the components poisoning the fermentation (Bothast, 1994).

Work performed during the current phase of this project led to the selection of one strain of *Pichia stipitus* which showed a conversion efficiency of 0.46 g ethanol per g xylose (Zhao and Dale, 1994- Appendix 2). These high conversion efficiencies however, were accomplished at a much lower specific ethanol production rate (gram ethanol per gram cell per hour) than is generally accomplished with *S. cerevisiae*. Thus, high cell densities will need to be maintained to keep the residence time under 24 hours on the two xylose fermentation stages. These xylose fermenting strains are much more strongly inhibited by ethanol as well, with a maximum ethanol tolerance of about 3%. We will continue to monitor the literature for organisms which might have performance advantages as compared to the strain we currently plan to utilize. Work during this project will include lab scale testing of cellulose to ethanol process design and testing followed by pilot scale implementation of the process. During the final 3 months of the project, the 15,000 liter unit will be operated on pretreated corn stalks.

Drawing Title:
Figure 3 BIOMASS PROCESS

Drawing No.
SCHEM6

Scale: NONE Date: 3/13/95

Issue: Drawn By: BLK

BPI Bio-Process Innovation, Inc.
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The Continuous Stirred Reactor Separator (CSRS) consists of stirred reactors matched with gas-liquid contractor stages. Removal of the ethanol during fermentation allows the fermentation rate to increase. Phase 1 work with the assistance of the NIST-DOE Energy Related Inventions Program led to the construction, testing and operation of a 24,000 liter pilot scale CSRS unit during 1994 with operation shown on starch and sugar streams. This CSRS is a new type reactor which allows simultaneous saccharification, fermentation, and ethanol separation in a combined process. Combining these reactions allows significant improvements in each operation. To operate the CSRS on biomass rather than starch, two cultures will be maintained within the reactor, a xylose fermenting yeast strain on stages one and two, and glucose fermenting yeasts on stages three, four and five.

The reactor's separation stages between stages 3, 4, 5 and exit must be designed so as to not clog with cellulose fibers. We tentatively plan to use a sieve plate design with slightly reduced plate area between holes to keep solids from settling. Downcomers will also be designed to be clog resistant. The designs will be tested on a 6 liter glass lab-scale unit during months 1-12 of the project.

Enzymes

Cellulase and/or xylanase will be added to the stages to release the sugars from the biomass. If the enzymes must be purchased, and are not able to be recycled, enzyme costs become an expensive part of the fermentation process. Enzyme costs for biomass hydrolysis were estimated by Lynd et al, (1991) as falling from \$1.00/gal for a 24 hour hydrolysis, to \$0.20/gal for a 5 day hydrolysis. Fermentation costs were estimated to increase from \$0.20 to \$0.38/gal as fermentation time increased. These sorts of high enzyme costs are a significant barrier to commercial implementation of enzyme based biomass to ethanol processes. In informal verbal conversations with H. Pesonen of ALKO LTD. (a Finnish company making and selling grain to ethanol fermentation systems), Mr. Pesonen stated that in their biomass to ethanol efforts, they found it necessary to generate their own enzyme on site to allow economical enzyme conversion of biomass to ethanol.

In this project, we will prepare some of our own cellulase/hemicellulase from three to four different organisms. Preliminarily we plan to grow strains of *Trichoderma reesei*, *Thielavia terrestris*, and *Aspergillus niger* as these cellulase producing fungi have temperature and pH optimums for growth and productivity near to yeasts. Co-culture and pre-culturing media to develop cellulases will then be examined. Fed batch and induction systems as proposed by Hendy et al (1984), Mandels and Weber (1969) and McLean and Abear (1986) will be evaluated to maximize enzyme production. We also plan to preliminarily evaluate a thermophilic bacterium, *Acidothermus cellulolyticus* which has been shown to excrete thermotolerant cellulases and is being developed by NREL researchers. A recent patent (Tucker et al, 1992) describes the use of this organism to

produce thermotolerant cellulase enzymes. The enzymatic breakdown of cellulose/hemicellulose requires three basic types of cellulase, endoglucanase which breaks down the cellulose polymer from within the chain, exoglucanase which works from the ends of the cellulose polymer, and cellobiase. Preliminary process design calls for the continuous production of cellulase in the hydrolysate tank (Figure 3) by culturing the cellulase organisms along with the pretreated cellulose. The broth from the CRS containing the cellulase enzymes will be re-circulated to the hydrolysate tank, allowing the enzyme levels to build-up over time. The cellulytic organism, and optimal conditions in the enzyme generation stages will be designed during phase 1 and 2 based on experimental findings and performances on small scale tests. The possibility of separate production and or concentration of the cellulase to the hydrolysis tank will also be studied and economically compared to the purchase of commercial cellulase. If recycling or immobilization of the enzymes allows economical use of commercial enzymes then this option will be tested on the pilot unit. The possibility of co-culturing cellulytic molds with the fermentation media will be evaluated as will some preliminary trials with immobilized enzymes as per Chen and Wayman (1989).

2. Technical Advantages

Bio-Process Innovations Inc. has designed, modeled and patented the CSRS for the production of ethanol from various substrates. This novel reactor/separator is coupled with a solvent ethanol recovery system to give a low energy continuous process for the production of ethanol from starch or biomass. Combining reaction with separation allows the fermentation of concentrated streams even if the fermenting microbe is strongly inhibited by ethanol. Simultaneous saccharification and fermentation of polysaccharides such as hemicellulose and cellulose can be quickly completed in this bio-reactor/separator, with the fermentation coupled with ethanol removal. Combining these reactions within the reactor vessel allows significant improvements in each operation as both enzymatic and ethanol reactions are product inhibited.

The reactor-separator concept of this invention consists of stirred tank type reactors operated in series, with the liquid streams moving from tank to tank contacted with a stripping gas to remove the ethanol product. Saccharification (of both polymers such as starch and cellulose) is sped by the reduction of sugar concentration as the sugar is fermented to ethanol. Fermentation is sped by the removal of the toxic ethanol product, and ethanol purification and concentration costs are reduced by the enrichment of the ethanol in the vapor phase. The gas stream is co-current to the tank to tank liquid flow in the enriching section, and counter-current in the stripping section. The final effluent from the CSRS is ideally characterized by complete saccharification of all polysaccharides (hemicellulose and cellulose polymers), complete fermentation of sugars to ethanol and complete removal or separation of the ethanol into the gas phase.

A schematic of the CSRS process is shown in Figure 3 for a system for biomass conversion to ethanol as suggested in this proposal.

The current project has included process development and demonstration of a low energy Solvent Absorption/Extractive Distillation (SAED) system for the recovery of anhydrous ethanol from ethanol vapors leaving the reactor/separator. A diagram of the SAED system is shown in Figure 4. As part of the current project, non-toxic, non-volatile solvents have been screened and tested. We have determined a solvent mixture which will allow good selectivity for ethanol over water. Our calculations based on the properties of this chosen solvent mix indicate that anhydrous ethanol may be obtained from concentrated sugar feed streams with an energy consumption ranging from 22,000 btu/gal at 5% sugar feed to 5,500 BTU/gal at 30% sugar feed by using a temperature swing between the absorber and the reactor as shown in Figure 5. This corresponds to a savings of about 50% over conventional processing using the current industry standard of distillation and molecular sieve dehydration as also shown in Figure 5.

There are several basic methods for cellulose breakdown, strong acid, dilute acid, ammonia explosion, steam explosion, and peroxide extrusion. Acid (sulfuric or hydrochloric) can serve both for disruption and hydrolysis of the cellulosic polymers. Strong acid allows complete breakdown of the components in the biomass to sugars, but also requires large volumes of concentrated sulfuric acid and can result in the production of furfural, an inhibitory byproduct (Goldstein and Easter, 1992; Ladisch and Swartzkopf, 1991). Dilute acid allows reduced acid concentrations, but requires higher temperatures, and again gives furfural. Ammonia explosion is a process being promoted by AFEX (B. Dale et al, 1985; AARC Bulletin, 1994)) which uses a quick pressure reduction after soaking the biomass with liquid ammonia solution. This system does not directly liberate any sugars, but allows the polymers (hemicellulose and cellulose) to be attacked enzymatically and reduced to sugars. Steam explosion and peroxide extrusion are similar processes. Steam explosion is being developed by Stake Technology which involves extrusion of the biomass at a high temperature and pressure, while peroxide extrusion uses a chemical pretreatment along with extrusion to accomplish the same goal of breaking down the internal structure of the biomass fibers. Steam explosion and peroxide extrusion allow enzymatic hydrolysis of the cellulosic polymers.

The Xylan process, by eliminating acid hydrolysis eliminates the production of toxic byproducts or carbonization (acid burning) associated with conventional sulfuric or hydrochloric treatments. The Xylan Delignification Process (XDP) seems also to be simpler and lower cost than the ammonia explosion (AFEX process, Dale et al., 1985) in which a high pressure explosion chambers must be developed, tested and scaled-up. We will follow the XDP process with an enzymatic hydrolysis of the biomass polymers. Enzymatic hydrolysis is slower than

acid or caustic chemical hydrolysis, but does not require the large volumes of chemicals, chemical recovery or neutralization, or the plant to be constructed of special acid/caustic compatible materials. We feel that the mild conditions and low chemical costs associated with enzymatic hydrolysis will outweigh the advantages of chemical (acid or caustic) hydrolysis. Both concentrated and dilute acid processes for biomass conversion are being pilot tested by TVA and NREL researchers (Barrier et al, 1986).

3. Energy Impact

This project will have two basic energy impacts. First, the goal is to replace fossil/imported oil energy sources with renewable, domestic liquid fuel, i.e. ethanol as a gasoline extender/replacer. Secondly, the goal is to produce ethanol economically in an environmentally friendly and energy efficient manner from domestic cellulosic biomass sources. Currently, almost all ethanol in the US is made from corn/grain starch sources. There is currently excess grain producing capacity in the US which allows the use of this grain for ethanol production, but in the long run, as export markets improve, it may be more beneficial to use the grain for human consumption. Biomass resources in the US are enormous, and include paper wastes, urban lawn wastes, wood pulp, straw, corn stalks, saw dust, sugar cane bagasse, etc. There is also a fair amount of current research on production of biomass crops such as switch grass and alfalfa for biomass to ethanol processes with a recent issue of Biomass & Bioenergy devoted to research papers on dedicated biomass feedstock supply systems in the US (B&B 6:3 1994).

Our efforts in this project focus on energy and capital savings for a process involved with the conversion process of cellulose to sugars and ethanol. Energy savings are attained by combining the CSRS reactor concept with solvent absorption of the ethanol from the gas stream exiting the CSRS. The enrichment of ethanol in the vapor stream (with a concentration of 5 to 8 times higher than liquid) combined with a further enrichment of ethanol in the solvent absorber (limited water solubility in the solvent) allows highly concentrated ethanol to be easily recovered from the solvent in a solvent stripping column. An anhydrous ethanol product may be recovered from the solvent using a simple extractive distillation procedure using the same solvent used for the ethanol absorption. As the ethanol concentration in the stage from which the gas exits is allowed to increase, energy costs for ethanol recovery drop. A part of this years project involved solvent testing and selection, combined with design and installation of the SAED system.

4. Cost and Economic Considerations

Ethanol production in the USA offers a renewable source of liquid fuel produced within the borders of our own nation as well as offering a market for excess grain/biomass crop production capacity of the midwestern states. However, in order for the ethanol fuel industry to be able to expand without governmental subsidies, ethanol production costs must be reduced closer to the level of refined unleaded gasoline (\$0.55-\$0.75 per gallon). Ethanol production costs can be reduced via:

- 1) reducing costs of substrate
- 2) increasing the efficiency of substrate conversion to ethanol
- 3) reducing the energy costs for purifying and dehydrating the ethanol
- 4) reducing the amount of bottoms waters which must be treated
- 5) reducing the capital costs for the ethanol processing plant
- 6) reducing the labor for operating the ethanol plant

The reactor/separator technology as being developed in the current project can meet several of these goals, namely increased conversion efficiency, reduced effluents, reduced capital, and reduced labor. In this proposal, we are trying to meet the first goal: low substrate cost through the utilization of biomass/paper wastes. In this proposal we suggest the testing of the lab and a 15,000 liter pilot unit on cellulosic materials (corn stalks). We would hope that this project will lead directly to the implementation of cellulose to ethanol on full scale plant size using waste cellulose such as waste paper, separated paper/cellulose from municipal solid waste plants, field crops such as switch grass and corn stalks, and saw dust.

As our nations oil supplies are depleted, and clean air requirements are stiffened, the need for ethanol fuels is becoming a national priority. If, however, current high energy technology (utilizing coal fired boilers) uses 40 to 120,000 BTU's of coal energy to produce 84,000 BTU's of liquid fuel energy per gallon of ethanol, the net effect is to produce a lot of coal fired boiler stack gas emissions to reduce car exhaust emissions. The overall effect on the US environment of this trade-off is open to debate. Current batch fermentation technology for ethanol production from corn requires large scale operations (12-50 million gal/yr. of ethanol), a large capital investment (\$2-4.00/ annual gallon), and is energy intensive. About half of this energy is associated with drying and evaporating the stillage, and half with fermentation and distillation. Presently, ethanol production level in the USA is at about 1,100 million gallons/yr. The total market for ethanol as a 10% blend in gasoline would be 12 billion gallons. As more and more ethanol is produced, it is important to our net energy position that ethanol be produced domestically in an energy efficient fashion. It is also important to develop processing for alternative feed stocks, such as biomass crops, rather than corn. Ethanol fuel production can help utilize excess corn capacity

(perhaps 20% of the nations crop) but further ethanol production must use other feedstocks if corn and grain prices are to be kept within 30 to 40% of the current levels.

A preliminary cost analysis for the Xylan/BPI biomass ethanol process was prepared based on cost and power estimates on extruders from Insta-Pro Intl. (a manufacturer), and cost estimates for the CSRS based on model size estimates and design costs equations as presented by Wood (1980, Engineering Economics in the Chemical Processing Industry). This analysis is presented in Table 1 for four basic scales, 1 million gal/yr, 5 million, 10 million, and 50 million gallons of ethanol per year. Assumptions were based on biomass composition of 35% cellulose, 28% hemicellulose, 30% lignin, and 8% ash. Only the costs of pretreatment and fermentation were determined. We did not estimate the costs of other necessary auxiliaries associated with a biomass to ethanol facility (raw material, labor, storage facilities), as these costs would be site specific (based on the raw material). As per Table 1, a net cost of between \$0.28 and \$0.35/gallon was determined for the process due to required fermentation nutrients, pretreatment chemicals, and energy costs to distill anhydrous ethanol. Pretreatment chemicals were determined to cost about \$0.120/gal. During our year 1 investigations, the possibility of substituting lower cost chemicals (specifically ammonia for sodium hydroxide, and lower amounts of hydrogen peroxide) will be studied with the intent of bringing this cost down to about \$0.05/gal. Labor, raw material, and waste treatment costs were not determined as mentioned previously. However, even at the smaller scales evaluated, 1 and 5 million gal/yr, a net cost per gallon (for pretreatment, fermentation, and ethanol recovery) of \$0.302 to \$0.355/gallon of anhydrous ethanol was determined. If the ethanol is sold at \$1.05/gallon, a ROI of 61% can be determined at the 1 million gallon scale, and 140% at the 5 million gal/yr scale suggesting that the process should in fact be quite profitable, even when added costs for labor, materials handling/storage, and raw product are added. By-product sales of yeast, CO₂ and lignin could also help improve the bottom line of the bio-refinery. Dried yeast currently sell at about \$.30/# for feed purposes, while lignin (sodium lignate sulfanate) has a selling price of around \$0.16/# (Chem. Marketing Rep, 1994).

Section 5. Commercial Potential and Market Considerations

Biomass is a generic term for plant substances which can be harvested from crop and timberlands and used as a renewable source of organic chemicals. A major goal within the Dept. of Energy and US Dept. of Agriculture is the development of an economic and environmentally responsible bio-fuels program to replace or supplement the use of petroleum as a liquid transportation fuel. Each gallon of ethanol produced in the USA displaces about 0.7 gallons of petroleum on an energy value basis. Our nation is currently importing about 50% of our petroleum needs (Gov. Et. Coal., 1994) with 110 billion gallons of gasoline con-

sumed in 1992 (Biofuels Update, 1994). Non-food crops such as switch grass and poplars grown on ground not used for food crops could produce 190 to 270 million tons of cellulosic biomass, which could then be turned into 30-50 billion gallons of ethanol (Brower, 1994). The major goal of this project is to help develop cost effective technology for the conversion of cellulose to ethanol, to allow the substitution of a renewable resource, biomass, for a non-renewable resource, oil. There is also a national security issue, in that our nation is dependent on foreign oil for 50% of our oil needs. Renewable resources, within the bounds of our own nation, could replace these imports with ethanol, and provide many jobs in production, collection and processing of the biomass.

Ethanol production in the USA offers a renewable source of liquid fuel produced within the borders of our own nation as well as offering a market for excess grain/biomass crop production capacity of the midwestern states. However, in order for the ethanol fuel industry to be able to expand without governmental subsidies, ethanol production costs must be reduced closer to the level of refined unleaded gasoline (\$0.55-\$0.75 per gallon). We hope that this project will lead to the implementation of cellulose to ethanol processes on full scale plant size using waste cellulose such as waste paper, separated paper/cellulose from municipal solid waste plants, field crops such as switch grass and corn stalks, and saw dust.

In a recent RFP from the Great Lakes Biomass Council (1995) it is reported that the EPA mandates on reformulated gasoline should increase the demand for ethanol by over 600 million gal/yr. Also reported are the following benefits of ethanol production in the USA:

- 1) the GAO estimates that increasing ethanol production from the current 1 billion gal/yr to 2-5 billion gal/yr would increase net farm income by \$415 million over an 8 year period.
- 2) The Federal government would save approximately \$500 to 600 million/yr even if tax subsidies to ethanol are continued at current levels.
- 3) Utilization of agricultural wastes as a feed stock will reduce price pressures on higher value feedstocks (grains).

It is our hope that current large scale ethanol producers will be able to expand production using this technology- perhaps continuing using current facilities for corn to ethanol while adding on biomass to ethanol capabilities. We also plan to market the process to farm co-ops in rural areas where biomass production could supplement or replace traditional grain crops. Finally, we see a market in the Municipal Solid Waste (MSW) market where paper/cellulose are being picked up and recycled. Goodman and Walters (1991) show that about 66 million tons/yr. of paper and paperboard are estimated to be discarded into MSW in the year 2000 along with 19 million tons of yard wastes. If 30% of this stream were converted to ethanol at a conversion rate of .3# ethanol/1 pound biomass, these streams alone could give 2.3 billion gallons/yr. of ethanol. Agricultural

crop residues such as corn stalks, wheat straw, or cotton stems have been estimated to be about 812 million tons/yr. which if 30% utilized could give 22 billion gallons of ethanol. The possibility of biomass crops such as hemp, kenaf, or other highly productive crops grown on marginal lands could expand this biomass availability even more. We feel that this development and demonstration project will allow the demonstration of several symbiotic technologies. The Xylan pretreatment system allows simultaneous saccharification and fermentation, while the CSRS further improves the kinetics of fermentation by keeping the ethanol levels low, which is particularly important during the first xylose-fermentation stages. Xylose fermenting yeast show fairly complete inhibition at 30 g/l ethanol. We hope that this technology would be applied to rural small scale ethanol production from biomass crops in the mid-west states, ethanol from cane bagasse in southern states, ethanol production from paper/cellulose separated from municipal solid waste (MSW) streams and recycled paper streams, saw dust from saw mill operations, and similar projects. It is a major goal of BPI to develop cost effective, low labor, efficient ethanol production processes and to market-license this technology as quickly as possible.

There are several competing technologies/processes for both biomass pretreatment and biomass fermentation. Which of these biomass systems is eventually most widely used is purely a response of the market to the profitability of the process. The Xylan process, by eliminating acid hydrolysis eliminates the production of toxic by-products or carbonization (acid burning) associated with conventional sulfuric or hydrochloric treatments. The Xylan process seems also to be simpler and lower cost than the ammonia explosion (AFEX process, Dale et al., 1985) in which a high pressure explosion chambers must be developed, tested and scaled-up. We will follow the XPD process with an enzymatic hydrolysis of the biomass polymers. Enzymatic hydrolysis is slower than acid or caustic chemical hydrolysis, but does not require the large volumes of chemicals, chemical recovery or neutralization, or the plant to be constructed of special acid/caustic compatible materials. We feel that the mild conditions and low chemical costs associated with enzymatic hydrolysis will outweigh the advantages of chemical (acid or caustic) hydrolysis. The expense of purchased enzyme is one concern associated with this process, hence the possibility of co-producing enzymes at the plant rather than purchasing the enzyme will be a part of our research/economic investigation.

Fermentation processes for biomass hydrolysate are being developed by Quadrex, Inc. who suggest a capital cost of \$2.00 per gallon at the 10 million gallon scale for their process (Co. Lit. 1992). Our calculations indicate a capital cost of under \$1.00 per gallon at this scale for our XDP/CSRS process. Quadrex have a genetically engineered bacteria which is able to metabolize both glucose and xylose well (Bealle et al, 1991) but actual performance of the organism in biomass hydrolysate has not been good due to poisoning (Bothast, 1994). Ho et al. (1994) at Purdue are currently developing a yeast which can apparently fer-

ment both xylose and glucose if nutritional conditions are rich. We feel our yeast strain may be the best currently available, but we will certainly be watching for better and more robust fermentation organisms which could be used in our reactor system.

Section 6. Needed Support/Budget

This project holds the prospect of making a significant impact on the energy use during production of liquid fuels in the US. BPI is a very small company with little or no financial resources to develop some of the ideas discussed in this project proposal. Thus governmental assistance during this development/demonstration phase of the invention process is critical to the timely development of the technology. We feel fairly confident that the input of governmental funds at this stage of our project will eventually return many times this investment in infrastructure, jobs, and taxes paid to the US government.

Work Plan

This project is divided into two phases, 1) the basic phase consisting of laboratory studies and process design, and 2) an applied or demonstration phase focusing on operation of the 15,000 liter CSRS for the production of ethanol at Permeate Refining Inc, in eastern Iowa.

Basic Research-The basic research will be performed at BPI's laboratory in W. Lafayette, IN. Dr. Dale will directly oversee the development and testing of the cellulosics fermentations, while the actual experimentation will be performed by Mr. Zhao, a biochemical engineering researcher. Mr. Zhao will be working on this project on a 75% time basis developing enzymes, time/temperature extraction data, and improving conversion efficiencies for biomass to ethanol. During the current ERIP study, xylose conversion kinetics for several strains of yeast were determined and one strain singled out for our further use. Kinetic data obtained in the lab will be incorporated into a process model of the cellulose conversion/fermentation/ethanol separation in the CSRS fermenter by Dr. Dale of BPI who has extensive experience in modeling of fermentation and separation systems (Dale et al, 1990). There will be three parts to our basic process development research:

- 1) Cellulase/hemicellulase production and performance comparison- The enzymatic conversion of the hemicellulose and cellulose tends to be the rate limiting step during the simultaneous saccharification and fermentation of these polymers. Strains of cellulase producing enzymes, (*T. reesei*, *A. niger*, *Thielavia terrestris*, and *A. thermocellum*) will be compared in lab studies for cellulase and hemicellulase activity. Based on these studies, the continuous cultivation of cellulase by one of these organisms will be implemented on the pilot study. Commercial enzymes will be compared for cost and effectiveness in hemi- and

cellulose hydrolysis. These will be obtained from three major enzyme companies 1) Solvay, 2) Genencor, and 3) Novo.

2) Bench scale testing of proposed biomass fermentation process-

A 4 liter CSRS has been constructed and is currently being operated to identify possible process improvement which would expand the application of the CSRS technology. This unit may have to be rebuilt to handle the biomass as some clogging problems were noted with starch, and the biomass, due to its fibrous nature will show an even stronger tendency to clog a bubble plate type contactor. We plan to build a glass CSRS lab scale unit with sieve type gas-liquid plates. We will implement the best enzyme/temperature program as determined from our batch scale tests along with the two stage (hemi/xylose SSF followed by cellulose/glucose SSF) process as shown in Figure 3. This reactor system will be operated from months 6 through 12. Experiments designed to improve the performance of the entire process will be continued during the entire course of the project. In this phase of the research we will also be testing the use of liquid ammonium hydroxide to replace sodium hydroxide as a pre-softening agent. If successful, this will reduce the cost of pretreatment chemicals substantially, as well as reducing the salts in the stream, as the ammonia will act as a nutrient during the fermentation process.

3) Process modeling/economics- A detailed process model of the process will be constructed including kinetics of enzyme hydrolysis, yeast conversion kinetics, and reactor size and ethanol separation characteristics. This model will be used to help optimize operational parameters for both lab and pilot scale tests, and to design and determine economics of full scale biomass to ethanol plants on various substrates at various scales of operation.

Applied Research- A pilot scale 15,000 liter 5 stage CSRS will be built during months 12-16, and tested on cellulosic feed stocks at the ethanol plant in Hopkinton during months 17-24 of the project. Xylan Inc will provide 60,000 pounds (one semi truck load) of pretreated corn stalks. Dr. Dale of BPI will develop the detailed designs for the 15,000 liter, 5 stage CSRS system by month 12, using experience gained from the glass lab scale unit. The unit will be built during months 12-15 by Merrill I&S and installed by month 16. The CSRS will then be run on pretreated corn stalks, with 60,000 # of stalks provided by Xylan Inc. over a period of 3 to 6 months

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Appendix G.

Design of Pilot CSRS w/ SAED
Dale, Moelhman and Zhao (1995)

Design of a Pilot Scale Continuous Stirred Ethanol Reactor Separator with Solvent Absorption and Extractive Distillation

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ABSTRACT

An improved bioreactor has been developed to allow the high speed, continuous, low energy conversion of various substrates to ethanol. The Continuous Stirred Reactor Separator (CSRS) incorporates gas stripping of the ethanol from the active fermentation broth using a recirculating gas stream between cascading stirred reactors in series to reduce the ethanol concentration within the reactor. A high cell density is maintained on the fermentation stages using a strain of flocculant yeast. The effects of turbulence on floc pellet size was studied. Ethanol recovery from the stripping gas leaving the bioreactor will be by absorption using a hydrophobic solvent. Design of an extractive distillation process to remove the water from the solvent prior to ethanol stripping is discussed. Energy needs for this process have been calculated to be under 8000 BTU/gal steam use for a 25% sugar feed. A 24,000 liter pilot scale version of the bioreactor has been designed, built and installed at an Iowa ethanol plant site. This pilot scale CSRS was started as a cascade reactor using starch dextrins and candy rinse water as a feed in May of '95. The SAED system will be added to the system in the near future.

INTRODUCTION

Ethanol production in the USA can offer a renewable source of liquid fuel produced within the borders of our own nation as well as providing a market for excess grain production capacity of the midwestern states. However, in order for the ethanol fuel industry to be able to expand without stage and federal subsidies, ethanol production costs must be reduced closer to the wholesale cost level of refined unleaded gasoline (\$0.55-\$0.75 per gallon). Ethanol production costs can be reduced via: 1) reducing costs of substrate, 2) increasing the efficiency of substrate conversion to ethanol, 3) reducing the energy costs for purifying and dehydrating the ethanol, 4) reducing the amount of effluent 'bottoms' waters which must be treated, 5) reducing the capital costs for the ethanol processing plant, 6) reducing the labor for operating the ethanol plant and 7) increasing the value of the by-products from the ethanol plant. A number of these objectives can be met using a continuous reactor incorporating simultaneous separation of the ethanol from the fermentation broth.

BIO-REACTOR DESIGN

The Continuous Stirred Reactor Separator (CSRS) consists of stirred tank type reactors operated in series, with the liquid streams moving from tank to tank contacted with a stripping gas to remove the ethanol product has been designed and tested. A US patent [1] has been issued to Bio-Process Innovation, Inc

which describes this reactor technology. The CSRS reactor can allow simultaneous saccharification, fermentation, and ethanol separation in a combined process. Combining these reactions allows significant improvements in each operation. Combining reaction with separation allows the fermentation of highly concentrated streams of up to 50% solids [2]. Simultaneous saccharificationⁿ and fermentation of polysaccharides such as starch [3] and cellulose can be quickly completed in this bio-reactor/separator. Saccharification (of starch or cellulose) is sped by the reduction of sugar concentration as the sugar is fermented to ethanol. Fermentation is sped by the removal of the toxic ethanol product, and ethanol purification and concentration costs are reduced by the enrichment of the ethanol in the vapor phase. The gas stream is co-current to the tank to tank liquid flow in the enriching section, and counter-current in the stripping section. The final effluent from the CSRS should be characterized by complete saccharification of polysaccharides, complete fermentation of sugars to ethanol and complete removal or separation of the ethanol into the gas phase. A schematic of the 4 stage 24,000 liter CSRS pilot plant is shown in Figure 1, with the design flows and concentrations on the various stages.

Figure 1. Schematic and design flows and compositions in the 24,000 L CSRS and Solvent Absorber

Fermentation rates in a bio-reactor are, in general, linearly related to yeast density if 1) the cell viability is maintained, and 2) the cell performance is not limited by local (micro-scale) substrate or product concentration gradients [4]. However, if cells are immobilized in beads or in flocs, there may be a decrease in specific productivity due to diffusion limitations, both of substrate to the cell, and ethanol from the cells. Teixeira and Mota [5] show about a 50% drop in specific sugar uptake in flocculated cells as compared to free cells.

During a normal free cell batch ethanol fermentation of a 15-20% sugar solution, a seed of perhaps 1×10^7 cells (0.5 g/l) increases over the 30 to 60 hour fermentation to a density of 1.5 to 2.0×10^8 (12-18 g/l) [6]. Fermentation rates can be increased if higher cell densities can be maintained during the fermentation. There are three basic ways to maintain a high cell density in a bio-reactor; 1) recycle the yeast after a yeast recovery step such as settling or centrifugation, 2) immobilize the cells within the reactor, in or on a support media (i.e. gel beads), or 3) self-immobilize the yeast by using flocculant yeasts and a settling area before removing the reactor out-flow. Self aggregation of the yeast is perhaps the simplest and cheapest method for achieving a high yeast density in that no additional equipment or support media is required for the process.

Four strains of highly flocculent *S. cerevisiae* and *S. uvarum* type yeast were obtained from various culture collections. Lab scale tests were used to observe the flocculating characteristics of the strains and to compare the settling speeds and fermentation rates. One strain was selected as being slightly more efficient ethanol producer, and adapted for the CSRS application by operating a 1 liter continuous stirred fermenter (New Brunswick Multi-Gen) for 60 days of continuous culture. A 15% sucrose feed was fed to the reactor at a residence time of 15 to 20 hours. Floc pellets of approximately 2 mm to 3 mm diameter developed and were maintained in this bio-reactor. Cell settling was observed to be complete within 15 to 20 sec after stopping the agitation (a stirring speed of 200 RPM- one blade- was required to keep the cells suspended). A cell settling layer of 33% to 40% by volume in the reactor developed and maintained itself at this density over time. Cell density in the supernate was very low, with the supernate broth appearing sparkling clear. Cell density in the settled layer was measured at 60 to 75 g/l at 5 minutes. Average cell density in the reactor for this adapted yeast was 20 to 30 g/l. This adapted floc yeast strain was then used for seeding the 24,000 liter pilot plant.

A short study on the effects of reactor turbulence on the floc pellets of the adapted yeast was performed under continuous fermentation conditions. Different levels of reactor turbulence were maintained by varying the stirring speed in a standard 2 or 4 liter fermenter (New Brunswick and Braun respectively) fitted with either 3 (anaerobic study) or 4 turbines—6 bladed open style—(aerobic study) for 48 hours after which time a sample was taken for microscopic analysis using a Hauser hemocytometer to measure a typical floc pellet dimensions. Under anaerobic conditions, a typical pellet size was observed to fall from 2.4×3.0 mm (width x length) at 250 RPM ($Re = 30,000$) to 1.2×1.6 mm at 400 RPM ($Re = 50,000$) to 0.2×0.4 mm at 700 RPM ($Re = 87,000$) to 0.2×0.3 mm at 800 RPM (100,000 Reynolds). A similar experiment run under aerobic conditions (2 VVM) showed pellet size falling from 0.4×0.8 mm at 250 RPM ($Re = 40,000$) to 0.4×0.4 mm at 500 RPM ($Re = 80,000$), to 0.3×0.3 mm at 700 mm ($Re = 115,000$).

Finally, at 750 RPM (Re of 125,000), the flocs were disassociated into free cells. Interestingly, the cells would not re-aggregate when the stirring speed was reduced to 250 RPM, either under aerobic or anaerobic conditions.

ETHANOL RECOVERY VIA SOLVENT ABSORPTION AND EXTRACTIVE DISTILLATION

The CSRS can be coupled with an isothermal solvent ethanol recovery system to give a low energy continuous process for the production of purified ethanol. A solvent is used to absorb the ethanol from the gas stream exiting the CSRS. The solvent should have the following properties: 1) low vapor pressure, 2) high affinity and miscibility with ethanol, 3) non-toxicity of any solvent vapor carry-over to the microbes in CSRS, 4) low affinity/solubility of water in the solvent, and 5) low solubility of solvent in water. The ideal solvent would absorb only ethanol from the vapor stream, allowing an anhydrous ethanol product to be stripped from the rich solvent. However, the close similarity in size and polarity between ethanol and water results in solvents having the ability to solvate ethanol also dissolve some water. Tedder et al. [7] tested for direct solvent extraction of ethanol using solvents with a 3 to 5% water weight fraction in the solvent phase when contacted with an aqueous phase and suggested the re-use of the solvent as an extractive distillation agent (SEED process).

Each solvent can be characterized by an equilibrium distribution coefficient, for ethanol between the water/aqueous phase and the solvent phase ($K_{dc} = x_{e(aq)} / x_{e(soln)}$), where x_e is the mass fraction of ethanol in either phase. There is a similar distribution coefficient for water, K_{dw} between the aqueous and solvent phases. Dividing the ethanol distribution coefficient by the water distribution coefficient (K_{dc} / K_{dw}) gives a separation factor, α , which describes the relative affinity of the solvent for ethanol compared to water. Dodecanol, found by Minier and Goma [8] to be nontoxic in direct solvent extraction in-situ separation, has an ethanol distribution coefficient of .21, and an ethanol/water separation, α , factor of 21. Work by Kollerup and Daugulis [9] indicated that dodecanol was somewhat toxic to microbes. Research in our labs has shown that dodecanol in direct contact with immobilized cells was toxic to yeast, but when used as vapor ethanol absorber, the dodecanol vapors carried over to an immobilized cell type reactor were not toxic, with fermentation rates of immobilized cells stable over 4 to 6 days [10]. By not actually contacting the cells with an organic phase, toxicity problems and solvent loss into the water phase are both minimized. Solvents examined for in-situ or post fermentation ethanol extraction include alcohols, esters, ketones, phenols, carboxylic acids, chlorinated hydrocarbons, amines, phosphorals, aromatic hydrocarbons, and oils [11, 12, 13].

An anhydrous ethanol product may be recovered from the solvent using an extractive distillation utilizing the same solvent used for the ethanol absorption leading to a solvent absorption extractive distillation (SAED) process as shown in Figure 2 [14]. The extractive dehydration process uses the principle that the solvent enhances the volatility of water more than ethanol, reversing the relative volatilities of ethanol and water. The activity coefficient for water in the solvent can be determined by the solubility of water in the solvent. For a three phase system (vapor, aqueous, organic) we can write:

$$P_w = \lambda(aq) P_w^o x_w(aq) = \lambda(soln) P_w^o x_w(soln)$$

where P_w is the vapor pressure of water, $\lambda(\text{solv})$ is the activity coefficient for water in the organic solvent phase, $x_w(\text{solv})$ is the mole fraction of water dissolved in the solvent, and P_w^0 is the vapor pressure of pure water. For pure water in contact with a test solvent, this reduces to:

$$\lambda(\text{solv}) = 1 / x_w(\text{solv})$$

Figure 2. The Solvent Absorption- Extractive Distillation (SAED) process for ethanol recovery from gas streams

The liquid/vapor equilibrium relationship for water (x_w) and ethanol in a test solvent (linoleic acid) as determined by our own solubility studies [15] and as given by the ASPEN 2 model are plotted in Figure 3 on a solvent free basis along with a proprietary second solvent (Solv.2) currently under evaluation for the SAED process. There is good agreement between our calculated equilibrium data for linoleic acid and the equilibrium curve suggested by ASPEN 2. Dehydration of the solvent would involve distillation of the water from the mixture as shown in the McCabe-Theile type analysis of Figure 4 for linoleic acid (equilibrium line from our data). As per Figure 4, approximately 13 stages (7 stripping, and 6 rectifying) with a water reflux ratio of 0.9 will allow a 98% mole percent water product to be recovered as a distillate from the top of the column, and a bottoms product with only 2% mole fraction water remaining in the ethanol. Vapor flow up the column can be provided either with a reboiler, vaporizing ethanol from the solvent, or by introducing an inert gas, CO_2 , to move the vapors upwards. Introducing the inert gas allows the temperature of the column bottom to be held near the temperature of the feed stream, allowing a single regenerative heat exchanger to feed the system as per Figure 2. The effects of temperature and inert gas flow rate in the dehydration column have been modeled using the Aspen II simulation using linoleic acid (11 stages, feed on stage 6) as shown in Figures 5 and 6. Assuming otherwise constant operational parameters (an inert gas to solvent mole ratio of 0.2, a solvent return to the top of the dehydrator at 0.5 F_{solv} and a water reflux ratio of 1.7), the fraction of water recovered in the distillate increases from 95% at 160° F to 99.5% at 200° F as the operating temperature of the column increases, but the fraction of ethanol leaving in the top distillate

also increases, so that a lower fraction of the ethanol leaves with the solvent in the bottoms stream as seen in Figure 5. Figure 6 shows the effect of the inert gas flow in the dehydration column at a feed/bottoms temperature of 170°F. If no inert gas is fed, very little water vapor exits the top of the column, and as the ratio of inert gas to solvent increases, the amount of water removed from the solvent increases, but the amount of ethanol leaving with the distillate also increases. A 98% removal of the water in the distillate, and a 94% recovery of the ethanol in the bottoms is obtained at an inert gas ratio of 0.2. After the water is removed from the solvent, the ethanol is vacuum stripped from the heavy solvent in a solvent stripping column. The hot solvent from the bottom of this solvent stripper is then taken back to the regenerative heat exchanger, cooled and recycled to absorb ethanol in the absorber again (Figure 2).

Figure 3. Water/Ethanol liquid vapor equilibrium in a hydrophobic solvent on a solvent free basis.

EXPERIMENTAL PERFORMANCE OF SAED SYSTEM

A six inch diameter column packed with Goodloe type net packing was built to test the performance of the SAED system. The extractive distillation column was 48" tall with approximately 18" of packing in the stripping portion of the column and 18" in the rectifying portion. Solvent with about 0.2-0.4% water, and 0.2-0.3% ethanol was fed to the system through a small plate and frame regenerative heat exchanger. Temperatures of 190-230 F were maintained using steam coils in the bottom of the dehydrator and vacuum stripper columns. Reflux ratios, temperatures, and flow rates were varied to test the ability of the dehydrator to distill water from the solvent, and the stripper to remove ethanol from the solvent. Table 1 gives our data on two runs. The results verify that the conditions identified in our hand and computer model can give good separations, with water taken as an overhead product, and ethanol as a bottoms. Ethanol levels in the distillate are higher than desired in Run 54 (20% vs. 5% w/v), but we feel that if there were more equilibrium stages in the dehydrator and vacuum stripper, results comparable to the model would be easily attained.

Figure 4. A McCabe-Thiele analysis of the extractive distillation of water from the solvent (solvent free basis)

Figure 5. The effect of operating temperature on the extractive distillation column based on ASPEN 2 analysis (11 stages).

NON-ISOTHERMAL OPERATION OF THE CSRS AND SOLVENT ABSORBER

Stripping efficiency of ethanol from the fermentation broth improves at higher temperatures due to the increased vapor pressure of ethanol, similarly absorption efficiency improves at lower temperatures due to the decreased vapor pressure of ethanol. Thus, if the stripping gas introduced to the CSRS is run warmer than the absorber the amount of gas recirculated can be reduced, leading to a reduced flow of solvent in the SAED system. For example, if the inlet gas to the CSRS is warmed to 42

Table 1
Experimental data from a 6" OD pilot scale SAED system.

Extractive Distillation (Dehydration Column)		
Solv. Feed Rate	Run 54 500 ml/min	Run 29 750 ml/min
Feed Solv. Composition		
ethanol	2.1 g/l	1.8 g/l
water	3.7 g/l	6.2 g/l
Feed Inlet Temp		
Bottoms Temp	215F	183F
Headspace Temp	231F	220F
Solvent Reflux Rate		
Water Reflux Rate	250 ml/min	75 ml/min
Inert Gas Rate	0.8 ml/min	0
Distillate Composition		
ethanol	12.7 l/min	6 l/min
water	0.8 ml/min	0
rate	933 g/l	67 g/l
Bottoms Composition		
ethanol	3.1 ml/min	n/m
water	200 g/l	67 g/l
Bottoms Composition		
ethanol	1.8 g/l	1.5 g/l
water	1.0 g/l	3.0 g/l
Vacuum Ethanol Stripper		
Feed Temp	226F	170F
Column Temp below Feed	219F	205F
Column midpt Temp	213F	205F
Bottoms Temp	230F	220F
Cond. Temp	44F	37F
Inert Gas Rate	3.4 l/min	6 l/min
Vacuum		
	24.2" Hg	23.5 in Hg
Distillate Composition		
ethanol	93% ethanol	45% ethanol
water	743 g/l	60 g/l
rate	0.43 ml/min	n/m
Bottoms Composition		
ethanol	0.32 g/l	1.3 g/l
water	0.47 g/l	0.5 g/l

Figure 6. The effect of inert gas flow on extractive distillation performance at 170°F based on ASPEN 2 analysis.

Energy Use as Function of Feed Sugar

Figure 7. Steam energy requirements for SAED compared to a conventional distillation for fermentation broths with different initial sugar concentrations.

C, (with heat added by the blower and some steam) while the absorber is operated at 25 C, the gas flow rate can be reduced by 33% in the CSRS, and the solvent flow through the SAED system can be reduced by 55% as compared to a isothermal system running at 32 C. These reductions in solvent flow also give a concomitant reduction in energy requirements.

ENERGY REQUIREMENTS FOR THE SAED PROCESS

The energy use for recovery of an anhydrous ethanol product from vapors in contact with various concentrations of ethanol was estimated. These energy calculations were done with the following assumptions: 1) an approach temperature of 5° C is maintained in the solvent regenerative heat exchanger. The inlet temperature of the rich cool solvent is 32° C, with an outlet temperature of 94° C, inlet temperature of hot lean solvent is 100°, and outlet temperature is 37°, 2) the rich solvent is heated a further 6° C with steam to 100°, 3) the lean solvent is cooled from 100° to 37° C regeneratively, and then cooled 60 to 31°C. with cooling water, 4) a molar reflux water ratio (L/D) of 1.0 is used in the dehydrator, 5) a reflux ethanol ratio (L/D) of 0.05 is used in the vacuum stripper, 6) an inert (CO₂) mole ratio of 0.1 (mole CO₂/mole solvent) is used in both the vacuum stripper and dehydration columns, 7) there is a 5° difference in operating temperature between the absorber (30°) and the gas to the bottom of the CSRS (35°C), 8) the vapor-liquid equilibrium for ethanol and water in the solvent is based on Figure 4, and 9) the inlet water concentration in the solvent is 0.4% by weight.

The calculated energy usages (based only on steam requirements) to recover an anhydrous product from feeds at various sugar concentrations are shown in Figure 7. These energy uses are approximately 55 to 66% of the energy costs associated with conventional distillation [16] combined with pressure swing dehydration (PSD) using molecular sieves. PSD was assumed to use about 1000 BTU/gal [17,18]. Energy requirements drop more or less linearly as sugar concentrations rise and higher ethanol concentrations are reached in the beer (an ethanol yield of 0.46 Y p/s was used in these calculations). However, once beer concentrations reach 10% (21% sugar) the

energy savings decrease slowly. Conventional brewing is generally limited to 10-12% ethanol to keep the length of brewing time low, while the CSRS can handle higher level sugars due to the gas stripping of ethanol.

THE PILOT SCALE CSRS

A 24,000 liter, four stage lab scale CSRS was constructed by Lamont Fabrication Co, Lamont IA. under the supervision of R.L. Lehman of PRI. The diameter of the column is 8 ft. and the total height is 22 ft. with the schematic in Figure 2. The ethanol vapors in the CO₂ stream leaving the CSRS are taken to the solvent absorber column. The absorber is rectangular in design and packed with a structured type packing to minimize gas phase pressure drop. A 10HP regenerative pressure blower from the New York Blower Company is used to recirculate CO₂ in the CSRS and absorber system.

The reactor was installed at the Iowa test site in March of 1995. The reactor was seeded and fermentation tests began in May. Nutrients (corn steep liquor and minerals) were metered using a peristaltic type lab pump. Problems were encountered with the seals leaking around the stirring shaft, which is of a single shaft design, going down the center of the CSRS. These seals were initially a block of Teflon with a close tolerance to the shaft dimension (2" diameter). This shaft leakage allowed the fermentation broth to drain from the top 3 stages without overflowing onto the gas-liquid contacting plates. Three different strategies for replacement of these seals with other type seals were tried in succession, until we moved to a 3" ID shaft sleeve/weir which was installed in August. The reactor was then reseeded and feed started on Sept. 8th. The reactor has been running continuously since this start-up through the current time (Dec. 1995), with no further difficulties with shaft leakage. Feed rates to the reactor range from 2 to 4 GPM at 20-23% sucrose, and flocculant settled cell densities (5 min) have been measured at between 5 and 30% v/v. Outlet sugar concentrations have been between 0 and 1% with ethanol concentrations of 9 to 11% ethanol determined.

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Appendix H

Performance of a 24,000 L CSRS.....
Dale, Gibb, and Lehman (1996)

PERFORMANCE OF A 24,000 LITER, MULTI STAGE CONTINUOUS STIRRED REACTOR SEPARATOR (CSRS) FOR ETHANOL FROM SUGARS, AND APPLICATION OF THE CSRS TOWARDS CELLULOSICS

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ABSTRACT

A pilot scale 24,000 liter, 4 stage reactor has been built, installed, and is undergoing performance trials. This Continuous Stirred Reactor Separator (CSRS) allows the continuous production and separation of ethanol from the fermentation broth using stirred reactor stages crossed with gas stripping of the overflow broth. The gas stripping of ethanol speeds fermentation rates by reducing the ethanol concentration on the lower stages. This CSRS is designed to have ethanol recovered from the exiting vapors using a solvent absorption column, after which the ethanol will be recovered from the solvent. The CSRS was installed in March of 1995, and has been operated as a continuous cascade type fermenter (without separation). The reactor has been running continuously since September of 1995 with feed rates varying between 0 (when feed tanks are exhausted) to 20 LPM. Yeast densities of a flocculent yeast have been measured at up to 50% cell settling volume. Preliminary work on biomass conversion to ethanol using the CSRS is being performed. Pretreatment studies, xylose fermentations, and SSF of cellulose have been started. It is planned to operate a two section CSRS for the successive conversion of hemicellulose/xylans followed by cellulose/glucans to ethanol.

Keywords: ethanol, bio-reactor/separator, biomass conversion

INTRODUCTION

Ethanol, produced by the fermentation of sugars, starch, and biomass streams, offers the promise of helping move the USA from a dependence on foreign oil towards a renewable source of liquid transportation fuel. However, to compete with oil, the cost of ethanol production should be in the same range as the traditional cost of refined gasoline, approximately \$0.55 to \$0.85/gallon. The performance of ethanolic reactors can be improved by three basic techniques: 1) move to continuous operations, thereby eliminating time spent emptying, rinsing, filling, and starting the fermentation, 2) increase the active cell density in the reactor as reaction rates are basically linearly related to the live cell density in the reactor, and 3) reduce the ethanol concentrations in the fermentation broth once the levels of ethanol are over 5% to 7%, allowing continued high rate fermentation of the remaining sugars. These techniques for reactor improvement may be met by a variety of processing technologies, but form the basis for the design of the continuous stirred reactor separator (CSRS). We hope the CSRS will allow lower capital and energy costs for ethanol and plan to develop a modular

technology which will allow the economic operation of smaller scale ethanol plants near sites of available carbohydrates.

The USA production capacity of corn is 6 to 8 billion bushels/yr currently, with ethanol production using about 5% of this capacity to produce about 4 billion liters of ethanol (USDA, 1994). Total demand for ethanol, if all gasoline were a 10% blend, would be 48 billion liters/yr. Thus, unless 50 to 60% of the total corn crop were devoted to ethanol, corn will not be able to meet the total demand for ethanol even as a 10% blend. Cellulosic materials in the form of straw, wood chips, waste paper, and corn stover should serve as a low cost source of feed stocks for ethanol production in the future. The amounts of cellulose available in the US is enormous, perhaps 85 million tons of paper and yard wastes from MSW alone. Crop residues of corn stalks, straw, and cotton stems are estimated to be about 812 million tons/yr. These sources at 30% utilization could produce 100 billion liters of ethanol. There are currently several technological barriers towards ethanol production from cellulose. We are in the process of applying the CSRS technology towards the production of ethanol from cellulose.

THE CONTINUOUS STIRRED REACTOR SEPARATOR (CSRS)

A modification of an immobilized cell reactor separator (Dale, 1984) was designed, moving from immobilized cells in a gas/liquid column, to a series of stirred tanks with the overflows contacted by a stripping gas. This CSRS concept was patented by BPI (Dale, 1992). The CSRS has two gas-liquid flow patterns, with gas moving co-current in an enriching section and counter current to the liquid overflows from the cascaded stirred reactors in a stripping section. The CSRS allows the introduction of insoluble solids with the feed. The CSRS can thus allow simultaneous saccharification, fermentation, and ethanol separation in a combined process, which allows significant improvements in saccharification and fermentation rates. Combining reaction with separation allows the fermentation of highly concentrated streams of up to 50% solids (Dale et al., 1994). Simultaneous saccharification and fermentation of polysaccharides such as starch (Dale et al., 1995) or cellulose fibers (not yet demonstrated) can be quickly completed in this bio-reactor/separator. Saccharification (of starch or cellulose) is sped by the reduction of sugar concentration as the sugar is fermented to ethanol. Fermentation is sped by the removal of the toxic ethanol product, and ethanol purification and concentration costs are reduced by the enrichment of the ethanol in the vapor phase. The final effluent from the CSRS should be characterized by complete saccharification of polysaccharides, complete fermentation of sugars to ethanol and complete removal or separation of the ethanol into the gas phase.

Operation of a 24,000 Liter CSRS

A 4 stage CSRS with a liquid fermentation volume of about 24,000 liters was designed by Dale of BPI, with R. Lehman of Permeate Refining Inc. (PRI) overseeing the construction and installation of the unit. A schematic of the unit is shown in Figure 1. The CSRS is 23 feet high by 8 feet in diameter. The first two stages are the enriching portion of the unit, while the second two stages are the stripping portion of the unit, characterized by countercurrent gas-to-liquid flow. Feed and nutrients are introduced to Stage 1 in which flocculent yeast are maintained.

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Figure 1. Schematic, design flows and compositions for the 24,000 L CSRS with solvent absorber.

The pH is controlled via individual pH controllers on each stage, which introduce aqueous ammonia when the pH drops below the set point. Temperature is controlled using self actuated temperature control valves which control the flow of cooling water through the coils on each stage. The reactor was installed in March of 1995 at PRI's plant site in Hopkinton IA. PRI operates a batch ethanol plant at this site with a nominal capacity of 8 million liters/yr of ethanol using food processing wastes as substrate (starches, candies, sugars and whey permeate).

Ethanol is separated by circulating CO₂ with a 10 HP pressure blower to the CSRS, where ethanol is stripped from the fermentation broth. The ethanol vapors are then adsorbed in a rectangular absorber using a heavy solvent as a selective agent. A process for extractive distillation of the co-adsorbed water from the solvent has been designed and tested on the lab scale (Dale, 1993, Dale et al., 1996), and the columns for ethanol recovery and purification were installed and plumbed during spring of 1996. As of this writing, we are

beginning shutdown trials of the system. The reactor has been operated as a cascade continuous reactor (without separation) since initial trials began in May, 1995. Yeast were grown aerobically in a non-sterile 1000 gal cone bottomed tank, with air fed to the base of the tank at 100 to 200 CFM with approximately 500 gallons of a 2 to 4% glucose media. The yeast were then pumped over to stage 1 of the CSRS which was filled approximately 25% with 20% feed. The feed to stage 1 was then started and the further stages inoculated by overflow as the reactor filled. Feed to the reactor consisted of two basic substrates, starch dextrin or candy rinse water from a Chicago candy manufacturer. Our desired feed concentration was 20% fermentable sugars or dextrans. During feeding with dextrans, gluco-amylase was metered to the reactor, along with nutrients (corn steep liquor and minerals).

During initial shake-down trials between May and August of 1995, problems were encountered with the seals leaking around the stirring shaft, which is of a single shaft design, going down the center of the CSRS as per Figure 1. The seals were initially a block of Teflon with a close tolerance to the shaft dimension (2" diameter). Shaft wobbling caused the seals to wear quickly. This seal failure allowed the fermentation broth to drain from the top 3 stages without overflowing onto the gas-liquid contacting plates. Three different strategies for replacement of these seals with other type seals were tried in succession, until we moved to a 3" ID shaft sleeve/weir designed by R. Lehman and installed in August. The reactor was then rinsed, reseeded and feed started on Sept. 8th.

An adapted strain of flocculating type *S. cerevisiae* has been developed by BPI. In lab scale trials, small pellets of 2 to 3 mm diameter were observed to form, with the pellet size decreasing with increasing reactor stirring speed (Dale et al., 1996). This yeast strain was used exclusively in the pilot scale trials. During the first several months of operation, feed cell densities on stage 1 ranged from 8 to 26 x 10⁷ ml⁻¹, but few flocs or measurable cell settling was noted. Lab scale tests showed that flocs could form in the candy water feed, but that the flocs deteriorated over time. It was hypothesized that perhaps the microscopic debris noted in the candy water inhibited or weakened the ability of the yeast to form flocs and/or pellets.

Stage pH's, stage temperatures, microscopic free cell count, and microscopic live free cell, and microscopic bacterial count were determined daily on each stage. pH was controlled at approximately 3.7 on stage 1, with pH's recorded ranging from 3.2 to 3.8. pH in the stages 2 to 4 were generally noted to stay within 0.5 pH unit of the pH of stage 1. A desired temperature profile of 30, 28, 26, 24°C on stages 1 to 4 respectively was occasionally attained based on a desire to maximize cell viability (Dale et al., 1990), but temperatures ranged from 23 to 30° on the stages. Performance of the CSRS operating as a cascade reactor over the period of 9/6 through 6/15 are shown in Table 1 with sugar (brix) concentrations measured with a hydrometer, with 2 to six data sets per month presented.

Table 1. Performance of Pilot CSRS Run in Cascade Mode.

Date	Feed (LPM)	Feed Brix (Percent)	Brix #1 (Percent)	Brix #2 (Percent)	Brix #3 (Percent)	Brix #4 (Percent)	Eth. #4 (% vol)	Floc #1 (% vol)	Floc #4 (% vol)
9/19/95	12	21	14	10.5	7	3	12.2		
9/20/95	12	21	9.5	6	4	2.4	12.3		
10/3/95	6	21	18	13	7	1.5		3	3.4
10/4/95	6	21	11	7	4	0			7
10/9/95	0	21	0	0	0	0	12		
10/10/95	20	15	2	1	0	0	12		3
10/25/95	6	21	7.5	3.5	1.5	0	10.6		
10/26/95	12	21	13.2	9	4	0.5			
12/18/95	0	22	4	3	0.5	0	11.6		40
12/19/95	12	22	8	6.5	3	0.5	11		30
1/12/96	12	22	13	8.5	5	3			
1/13/96	0	22	10.5	8	4.5	1	10.2	10	30
1/26/96	8	22	17	14	10	6			
1/30/96	8	22	12.5	8.5	5	2			
2/29/96	16	24	15.5	12	9.5	9	7.6		
3/1/96	0	24	5.5	4	3	1	9.8		
3/18/96	0	21	7	10	9.5	3.5			
3/19/96	10	21	9.5	6.5	6.5	6	8.8		
4/9/96	4	21	5	0	0	0		30	20
4/10/96	0	21	4	0	0	0		30	25
4/23/96	0	19	1	1	1	0.5			
4/24/96	8	19	6	1.5	1	1		24	21
5/14/96	12	20	9.5	5	3	1.5		10	32
5/15/96	12	20	9	2	1	1		4	27
5/24/96	12	20	9.5	6.5	3	1		44	40
5/28/96	16	22	16	14	11	8		5	20
6/4/96	12	22	7	5	2.5	1		50	50
6/5/96	16	22	12	7	4	2		40	40

As per Table 1, when feed was stopped due to the feed tank being exhausted, the brix on each stage would go to near zero (10/9/95, 4/9, 10, 23/96). The feed rate was controlled by a ball type valve on the outlet of a centrifugal pump. Occasionally the feed rate would go higher than desired, with the result that brix levels rose and cell concentrations dropped (2/29/96, 5/28/96). After these events, feed was stopped until a brix of under 1 was noted on stage 4 after which the feed was restarted. The reactor showed a remarkable ability to recover from both feed stoppages and feed oversupplies. A desired brix profile of 12, 6, 3 and 0 was often nearly attained.

Stage 1 of the reactor was re-seeded with the flocculent yeast on 12/8/95, as lab studies on the strain had shown that when the cells were deflocculated by high shear in aerobic conditions, they would not reaggregate no matter what conditions were tried (Dale et al., 1996). Some good flocculation was noted almost immediately on 12/18/95. Flocs persisted through mid January, after which none were noted until April. Better flocs were noted with dextrin feeds than with candy rinse water. The adapted flocculent yeast seems to be very resistant to bacterial (or wild yeast) contamination. Although care was exercised in the propagation of the yeast, and the reactor was manually rinsed with a sanitizer

prior to seeding, none of the operations were sterile. Thus, the yeast were not propagated under sterile conditions, the reactor prior to start-up was not sterilized, nor is the feed to the reactor sterile. Despite these continuous challenges, few bacteria were noted. A bacterial density of $4 \times 10^7 \text{ ml}^{-1}$ was noted on 10/4 and 2 to $3 \times 10^7 \text{ ml}^{-1}$ on 12/18/95. Most days the bacterial visual count was under 1×10^7 . Contamination problems were also determined to be minimal during our lab adaptation work with this yeast. We hypothesize that this yeast may have some mild anti-bacterial activity.

The CSRS for Ethanol from Biomass

Different cellulosic materials have different levels cellulose, hemicellulose and lignin, with cellulose ranging from 30 to 40%, hemicellulose from 18 to 25%, and lignin from 19 to 23% for typical biomass sources. There are two basic methods for conversion of biomass to fermentable sugars, acid hydrolysis and enzyme hydrolysis. Most large scale efforts at biomass to ethanol are currently focusing on acid hydrolysis, but there is some feeling that the enzyme conversion treatment will eventually be more popular due to the lower use of chemicals and milder operational conditions. The conversion of biomass (through enzymatic techniques) to ethanol consists of four basic steps: 1) pretreatment of the biomass to allow enzymatic attack of the bio-polymers, 2) conversion and fermentation of the hemicellulose/xylose fraction, 3) conversion and fermentation of the cellulose/glucose fraction, and 4) separation and concentration of the ethanol. We are focusing on an enzymatic, two stage conversion process of biomass to ethanol, using the CSRS reactor technology to speed fermentation rates. We suggest dividing the CSRS in to two basic sections, with the first section containing stages devoted to the xylose fermentation using a xylose fermenting yeast, followed by a second section devoted to the cellulose/glucose fermentation. In order to bring this concept to demonstration, there are three basic goals: 1) establishment of an economical and effective pretreatment process, 2) improve the xylose to ethanol fermentation, and 3) demonstration of SSF of the cellulose fibers in the second section of the CSRS.

Pretreatment: Two types of pretreatment are being evaluated: 1) the Xylan Delignification Process (Dale et al., 1995), and 2) a basic steeping process. We found that the XDP process allows good enzymatic attack of the biomass with near complete release of glucose/cellobiose from cellulose and xylose from hemicellulose/xylans within 7 to 10 hours. In preliminary studies, we have noted that a steeping process in a basic medium also gives excellent solubilization of lignin and hemicellulose. We will be comparing these processes on a fermentability and economic basis during the next year.

Xylose Fermentation: Strains of yeast able to ferment xylose to ethanol have been studied by various researchers (Slininger et al., 1985, Laplace et al., 1993), and genetic manipulation of both yeast (Ho et al., 1994) and bacteria (Bealle et al., 1991) have led to xylose to ethanol fermentations. We have evaluated a number of strains of xylose to ethanol yeasts, and determined that strain *P. stipitus* NRRL 11545 was advantageous when both glucose and ethanol were present (Zhao and Dale, 1995). We have recently adapted a strain of flocculent *P. stipitus* for the CSRS derived from *P. stipitus* NRRL 7124. A 1 liter stirred reactor was used to adapt the strain. The reactor was started with an 8% xylose feed for days 1 to 22, and 8% glucose from days 23 to 45. Conditions promoting flocculation were studied. An aeration rate of approximately 1% was used with a stirring speed of 150 to 250 RPM. Aeration with a micro-porous sparger (small bubbles) seemed to promote deflocculation and move the yeast as a scum on top of the

fermenter broth. Brix and settling volume (5 min) of the reactor are shown in Figure 2. By 35 days of operation, a settling volume of 30 to 40% was regularly attained in the reactor. Specific rates of ethanolic fermentation of glucose by *Pichia stipitis* are 2.5 to 3.5 times faster than on xylose under the same conditions (Grootjen et al., 1990).

Cellulose Fermentation: The Simultaneous Saccharification and Fermentation of cellulose to ethanol has been widely studied. High temperatures improve the performance of cellulase, so use of a temperature tolerant yeast strain is useful. Dale et al., (1995) used a temperature tolerant strain of *K. marxianus* to demonstrate SSF of starch in a CSRS. Some preliminary trials of batch SSF of XDP pretreated corn stalks and switch grass have been performed (Dale et al., 1995). These tests showed that reasonably good conversion of the cellulose could be attained using a D5A strain of *S. cerevisiae*. These initial trials showed 50 to 80% conversion of the cellulose to ethanol, and better yields of 85 to 90% are expected to be attained.

Figure 2. Development of a Flocculant Xylose Yeast

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