

ENZYMATIC SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF) OF BIOMASS TO ETHANOL IN A PILOT 130 LITER MULTISTAGE CONTINUOUS REACTOR SEPARATOR

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ABSTRACT

BPI has designed, built, and is just beginning to run tests on a pilot scale process for cellulosic based ethanol using four new and proprietary technologies based on enzymatic hydrolysis of biomass. A 130 liter 6 stage continuous stirred reactor separator (CSRS) was constructed to convert sequentially both the cellulose and hemicellulose fraction of pretreated biomass to ethanol while simultaneously stripping the ethanol from the fermentation broth. BPI's technologies include: 1) a non-acid proprietary low temperature delignification process which has the advantage of giving a lignin by-product and reducing chemical needs for the pretreatment via chemical recycle, 2) the enzymatic Simultaneous Saccharification and Fermentation (SSF) of the cellulose/glucans fraction of the biomass in the first three stages of the CSRS at 42C uses a thermo-tolerant strain of yeast, and 3) the subsequent fermentation of xylans using a strain of *Pichia stipitus* at 30 C in the final 3 stages of the CSRS, 4) the simultaneous stripping of the ethanol from the fermentation broth, and 5) the recycle of a large fraction of the enzymes/ fermentation broth to the reactor/separator to react with fresh pretreated biomass.

BPI has done pretreatment studies on a variety of biomass substrates; straw, corn stover, hardwood shavings, and oat hulls. Oat hulls (which have a composition very similar to straw) will be pretreated using BPI's delignification process to provide biomass for the pilot scale operations.

Keywords: biomass, cellulose, ethanol, enzymatic SSF, Multi-stage reactor/ separator, xylose fermentation

Introduction

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 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 develop an economically profitable process for the production of ethanol and co-products 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 current research on production of 'dedicated' biomass crops such as switch grass and alfalfa as feedstock for ethanol processes in the US (Biomass & Bioenergy 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. These barriers have prevented the commercialization of biomass to ethanol projects in the US to date. We feel as though BPI has an excellent set of technologies which should pave the way for economic production of ethanol from biomass.

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 commercial cellulase 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 (sugar) inhibition. This simultaneous saccharification and fermentation (SSF) is thought to be 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 (being developed by Xylan Inc.) 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. BPI is in the process of developing its own (low temperature steep delignification) biomass pretreatment process.

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. BCI, with the help of the DOE, is constructing a plant in Louisiana for bagasse conversion to ethanol using acid hydrolysis and some genetically engineered bacteria.

Ethanol production from cellulose using enzymatic hydrolysis can be improved by:

- 1) Development of pretreatment technology which is effective, does not require expensive chemicals and/or high pressure expensive equipment
- 2) Maintaining a high density of cells within the reactor so as to convert sugars to ethanol quickly...glucose levels should be kept below 2 g/L
- 3) Combining enzymatic conversion of cellulose and hemicellulose polymers with fermentation so as to keep sugar levels low, improving enzymatic conversion rates by minimizing product (sugar) inhibition of enzymatic conversion of the biomass to sugars.
- 4) Converting both the cellulose (glucose) and hemicellulose (xylose) to ethanol

- 5) Co-producing crude cellulase enzyme and/or recycle the enzymes so as to reduce enzyme costs.
- 6) Incorporation of low temperature separation of ethanol from the reactor broth so as to keep fermentation reaction rates high, and allow recycle of enzymes w/o thermal destruction.

The central effort of this project is to develop and demonstrate a continuous multistage stirred reactor separator for the successive fermentation of glucans and xylans which incorporates all of the above design parameters improvements. Biomass generally consists of about 25-30% lignin, 25-30% hemicellulose, and 30-45% cellulose. The ideal process for biomass conversion to useful products must allow utilization of each of these three major biomass components.

BPI is developing a delignification pretreatment process, which is followed by enzymatic release of xylans and glucans from the hemicellulose and cellulose respectively. Combining reaction (fermentation) with enzymatic release of sugars (Simultaneous Saccharification and Fermentation or SSF) improves the enzyme kinetics due to reduction of product inhibition. Further combining ethanol separation from the reaction improves the fermentation rates of the yeast. The basic process flows of the CSRS process for cellulose is shown in Figure 1. BPI is pursuing a dual fermentation of the biomass sugars. First SSF of the cellulose at 42C using a thermotolerant strain of *K. marianus*, followed by xylose fermentation using a strain of *P.stipitus* (Zhou & Dale, 1994). There is an effort by three different groups in the USA- Purdue University (Ho et al. 1997) with a *Saccharomyces* yeast, University of Florida (Bealle et al, 1991) with an *E. coli* bacterium, and NRRL with a *Z. mobilis* bacterium- to develop a single microbe which can ferment both glucose and xylose. However, these organisms while having a bright promise, tend to be genetically unstable and not to have the temperature tolerance we are looking for in our project. The optimal activity for cellulase is about 45C. We feel that the dual strain, sequential fermentation of the glucose and xylose as proposed here is the best option for near term commercial production of ethanol from biomass.

1. Pretreatment Studies

BPI has been developing a 'steep delignification' process which solubilizes lignin using a proprietary process. The pre-treated biomass is then 'available' for enzymatic attack of the cellulose and hemicellulose polymers.. Our selection of treatment chemicals allows their recovery and recycle as per Figure 1.

BPI's pretreatment process consists of a low temperature (ambient), low pressure (atmospheric), steeping process where the biomass is delignified using a proprietary non-acid blend of chemicals. We have evaluated four types of biomass to date:

- a) cut straw
- b) corn stover
- c) hardwood shavings from a furniture factory
- d) oat hulls.

We have found that our process works well on all of these types of biomass.

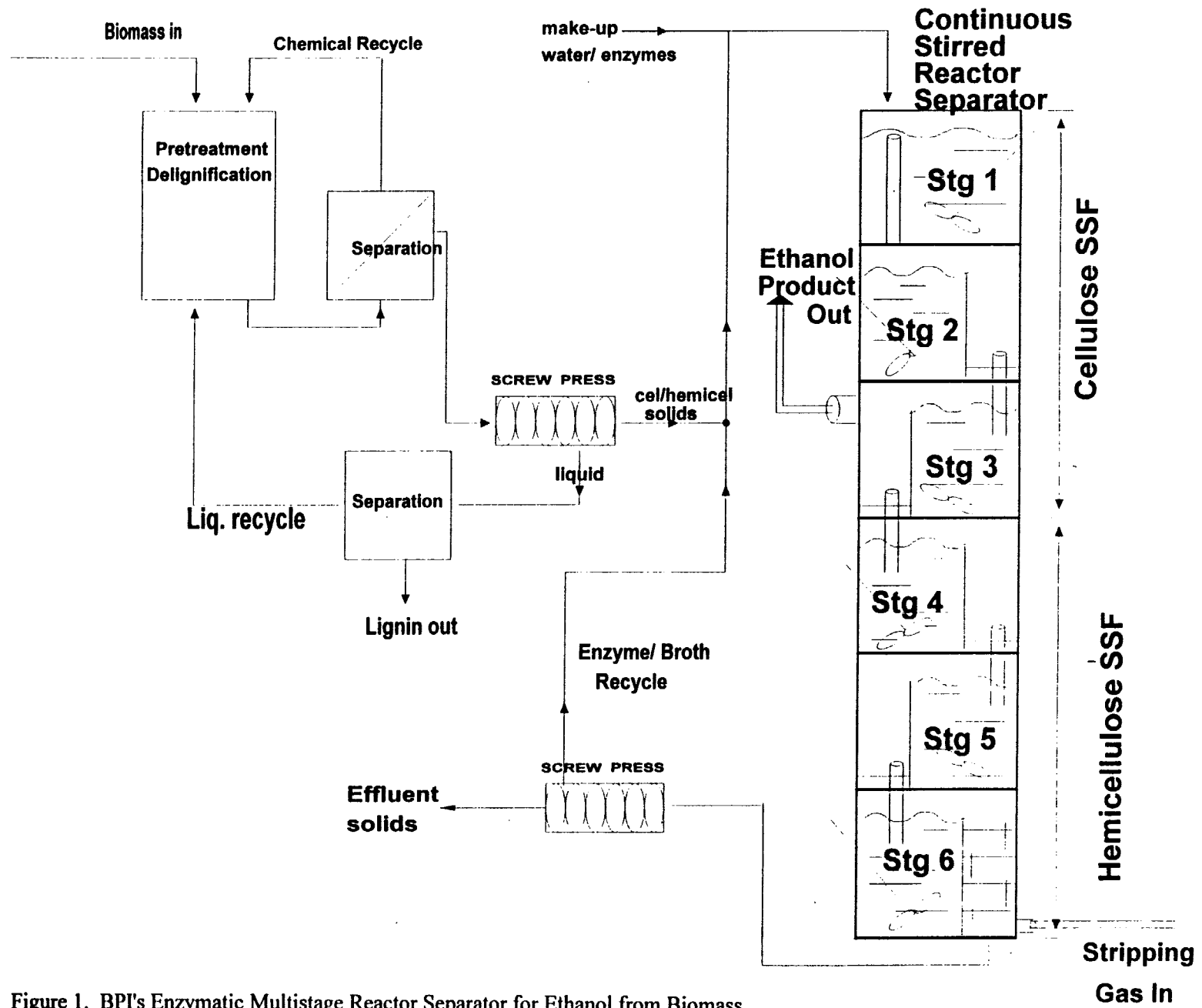


Figure 1. BPI's Enzymatic Multistage Reactor Separator for Ethanol from Biomass

Straw/ Oat hulls- Our most recent studies have indicated that excellent conversion of the biomass (125 g/L biomass) can be attained with a simple ambient steeping. Recent work on determining optimal (i.e. minimal) levels of pretreatment chemicals resulted in xylose release during the SSF of the cellulosic portion of the biomass. We have been routinely seeing about 30 g/L of free xylose at the completion of our batch bench scale SSF's of pre treated biomass (125 g/L biomass). This compares to previous results of 5 to 10 g/L xylose seen in the completed fermentation (glucan/ethanol) at higher levels of pretreatment chemicals and longer steeping times, indicating that those conditions, the xylose/xylans were being released into the pretreatment broth, prior to separation and SSF of the residual delignified biomass. In Figure 2, a sample batch SSF of straw is shown.

Corn Stover- We found that corn stover had very similar performance to straw in our pretreatment/ batch SSF studies.

Hardwood Shavings- Using maple shavings from an Indiana furniture manufacturing plant, we found that simple steep delignification gave poor performance as far as released sugars and ethanol in our batch studies. Agitation and physical mixing during the pretreatment were required before similar (25-30 g/L) ethanol levels were obtained during the SSF (cellulosic @ 42C) of the pretreated hardwood at 125 g/L solids. A sample batch SSF of hardwood shavings is shown in Figure 3.

2) Enzymatic Simultaneous Saccharification and Fermentation (SSF) of the cellulose fraction of pretreated biomass

The enzymatic conversion of cellulose is usually the rate limiting step during the simultaneous saccharification and fermentation. We had completed trials during 1998 using a cellulase product from Solvay Enzyme, *TRL Cellulase*. With this enzyme we noted good residual enzyme activity leading us to our process design using recycle of fermentation broth/enzymes, so as to minimize fresh enzyme needs. However, Solvay Enzyme was acquired by Genencor in 1999, and we then obtained samples of two enzyme products from Genencor, *Speczyme CP* and *Speczyme CE*. The Genencor cellulases however, were determined to 1) have some toxicity to yeast, and 2) to have very little residual enzymatic activity after a single SSF. Thus these enzymes performed substantially differently (worse for our purposes) than the Solvay *TRL* product. We then evaluated cellulases from a number of commercial producers.

- 1) **Valley Research Inc.-** sent: "*Validase TR Concentrate*", and "*Cellulase 4000*". Costs of their product ranges from \$33-\$48/kilo in 25 K lots. They also sent 4 'experimental' cellulases which we have tested for suitability on pretreated biomass
- 2) **Enzyme Development Corp.** sent samples of a cellulase product called "*Enzeco Cellulase CEP*".
- 3) **Amano Enzyme** Their enzyme products retail for around \$38/Kilo for 30,000 unit/g. They sent samples of, "*Cellulase A- 3*", "*Cellulase T- 4*", and "*Cellulase AP-10*".
- 4) **Dyadic** sent a sample of their cellulase product called "*Rocksoft ACE*"

Figure 2.
SSF of SD Treated Wheat Straw

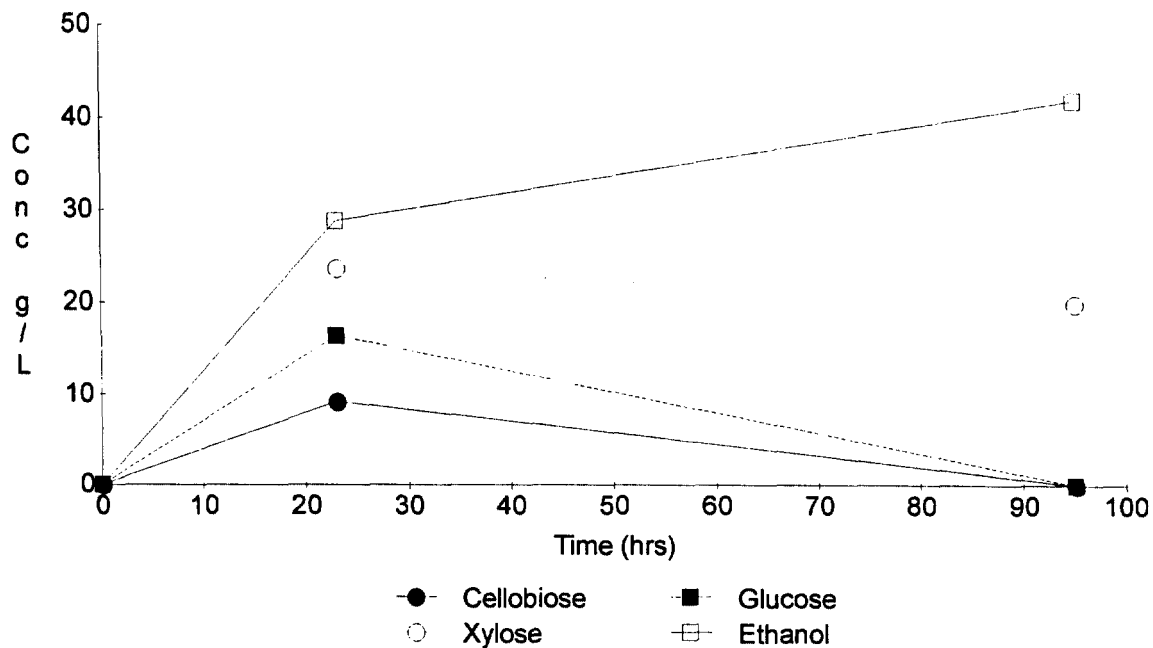
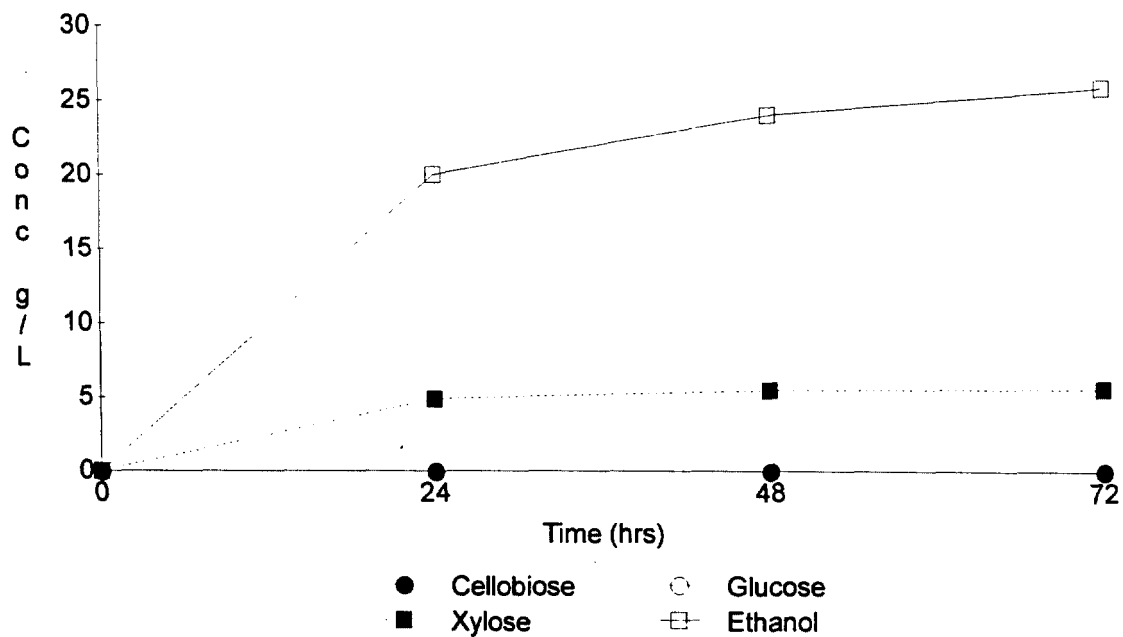


Figure 3.
SSF of Maple Shavings



Activities for these enzymes is given in a variety of units as defined by the manufacturers. Each company has devised their own method of measuring cellulytic activity units. Thus, it is difficult to know or guess how the enzyme will perform on biomass. In general, *T. reesei* derived enzymes are better suited for biomass breakdown than are enzymes from *A. niger*. After receiving the enzymes from the various companies, we began SSF experiments on pretreated straw. We tried levels of enzymes ranging from 8 to 30% of the weight (d.b.) of biomass added to the fermenter.

Table 1. Enzyme Comparisons

Time	Dyadic "Rocksoft"	Valley R. "TR"	Valley R. "4000"
48 hours	still viscous	16.7 g/L ethanol	23.5 g/L ethanol, very thick
72 hours	still viscous	16.2 g/L	32 g/L, still very thick

Rocksoft @ 12%, TR @ 8%, 4000 @ 30% enzyme use level

Table 2. Enzyme Comparisons

Time	Amano "4"	Amano "AP10"	Dyadic "Rock"	Valley R. "TR & 4000"
24 hours	no liquifaction	no liq	13.5 g/L eth	no liq
44 hours	still viscous	slight liq	15.3 g/L eth	slight liq
70 hours	10.7 g/L eth	10.7 g/L eth	12.5 g/L eth	27.5 g/L eth

Amano "4" @ 3.2% d.b. biomass, Amano "AP10" @ 3.2%, Dyadic @ 124% TR&4000@ 6% & 8%

Table 3. Enzyme Comparisons

Time	Amano "AP10"	Dyadic "Rock"	Valley "TR"
72 hour	24.4 g/L eth	19.8 g/L eth	31.8 g/L eth

AP10@ 9.5%, Dyadic@ 3%, TR at 15%

Table 4. Enzyme Comparisons

Time	Amano "AP10"	Valley "TR"	EDC "CEP"
20	not liquified	15.7 g/L	32 g/L
44	still thick		
	27.2 g/L	24.7 g/L eth	38.7 g/L
70	heavy solids	26 g/L	32.6 g/L ethanol

all enzymes @ 7.6% enzyme to biomass d.b.

Based on these comparisons shown in Tables 1-4., Valley Research's "TR" cellulase, and EDC's CEP enzyme seemed to be most effective at quick and complete liquifaction at a fairly low use level. The Amano enzymes did not seem to be able to liquify well.

Residual Enzymatic Activity of Enzymes

The ethanol was stripped from the completed batch fermentation broth using a vacuum rotoevaporator at 50C. The stripped broth was then used to make-up a second SSF using fresh pretreated biomass. No further fresh enzyme was added.

a) Valley Research "TR" cellulase

In our recent work with Genencor cellulases, fairly good conversion of biomass to ethanol were achieved, but the residual fermentation broth after a batch fermentation had very little enzymatic activity remaining- contrasting with our earlier work with Solvay TRL cellulase which showed excellent residual activity.

The residual activity of Valley Research cellulase was evaluated with and without solids. (One theory as to the lack of cellulase activity of the Genencor enzyme was that the enzyme was terminally bound to the residual solids.) In this experiment, broth from a SSF of BPI pretreated straw (with an enzyme level of 16% d.b. enzyme/biomass) was vacuum stripped. Pretreated biomass (at 125 g/L) was then added to the stripped broth.

Table 5. SSF w/ recycled V.R. "TR" enzyme

time (Hours)	broth w/o solids	broth w/ solids
0	no ethanol	no ethanol
72	13.8 g/L ethanol	15.7 g/L ethanol
96	no sample	12.5 g/ L ethanol

In this experiment, good liquifaction and ethanol levels were noted in both samples with and without residual solids. (If a good liquifaction of biomass is achieved, little solids remain. This is one of the criteria used for selecting a cellulase in our evaluations.)

b) EDC "CEP" cellulase

A similar experiment with EDC's enzyme was conducted. All the broth (including solids) was recycled. Original enzyme concentration was 16%. Biomass at 124 g/L was added to the recycled stripped broth. Good liquifaction and ethanol production was noted with 22.4 g/L ethanol measured at 20 hours, and little further ethanol generation over the next 20 hours (22.9 g/L at 44 hours).

3) Design/ Fabrication of 130 L Multistage Continous Reactor/ Separator

A 6 stage MCSRS was fabricated from 304 stainless steel. Broth cascades from one stage, across a gas-liquid separation contactor and then into the next stage. The column diameter is 30 cm and the height of each stage is 30 cm. Total reactor volume is 130 L over the six stages. Gas (CO₂) is recirculated through the reactor, and then into an absorber, where it is contacted with water to absorb the ethanol vapors. The CO₂ is then recirculated back to the MCSRS. Figure 1 shows the basic layout of the 6 stages. We hope to be able to present performance data at the Bio-Energy Conference from the pilot MCSRS.

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