Modification of Biomass to increase Ethanol yield

Naresh K Budhavaram*, Aarti Gidh, Clint W Williford, Alfred Mikell
The University of Mississippi, MS – 38677

Introduction

Currently available petroleum is not only a non-renewable source of energy but also causes lot of pollution. With all the petroleum prices going up and petroleum reserves depleting, there is an urgent need of an alternative. Ethanol is one such alternative which can meet the growing demand for fuel. Ethanol is easily biodegradable and also causes less pollution. Ethanol can be produced chemically and biologically using agricultural products. Bio-Ethanol is traditionally manufactured from sugarcane and cornstarch. But the ethanol produced by this procedure is not only costly but also depletes food reserves (M.Clarke Dale & Mark Moelhman). Instead ethanol can be manufactured from lignocellulose obtained from agricultural wastes such as corn stover and grass. Processing just 30% of cornstover adds 5 to 8 billion gallons of ethanol fuel without any need to use more land (David Glassner, CTIC meeting, 1999).

The corn stover feedstock comprises of 37.5% cellulose, 22.4% hemicellulose and 17.6% lignin by dry weight composition (Mosier *et al*., 2005). Cellulose and hemicellulose polymers are the basic building blocks of the fibers while lignin is the binder and gives the structural strength (Sjostrom, E, 1981). Lignin binds the cellulose and hemicellulose and protects them from microbial attack. Due to this, lignin acts as a barrier to the conversion of cellulose to ethanol, hinders the cellulose and hemicellulose separation to sugars and also inhibits the fermentation process (Ramos *et al*. 1994).

At present to make cellulose accessible to enzymes the biomass is pretreated chemically. Different varieties of chemical pretreatment procedures are available such as dilute acid pretreatment. But there are many disadvantages to these pretreatment processes. The large quantities of chemicals used create large amounts of waste thereby causing waste disposal problems. Also these procedures consume large quantity of water. The yield of ethanol is also low. These factors account for the higher cost of ethanol.

As an alternative to the chemical pretreatment the use of biological agents such as fungi and bacteria is being investigated. Literature suggests that white rot fungi and bacteria can degrade/digest the lignin. This would separate cellulose from lignin. Our work mainly focuses on finding suitable organism which can separate cellulose and hemicellulose from lignin, thus enhance the susceptibility of cellulose to enzymatic attack, and ultimately increase the ethanol yield. In our work, we are trying to screen different wood eating insects. We have screened beetles and conducted weight loss experiments using different parts of the beetle such as foregut, mid gut and hindgut.

Materials and Method

Pretreated and untreated corn stovers were used as substrates. A total of fourteen sources from the foregut, midgut and hindgut of beetle were isolated and grown. The fourteen sources from the beetle gut were isolated and were allowed to grow in saline. Gram staining and Biolog tests were conducted on the grown isolates to characterize them. Biolog plates containing positive and negative stains were used. A Spectrophotometer was used to measure the cell density of the inoculum.

The pretreated and untreated corn stovers were ground into fine powder. Then water was added to cornstover and was bought up to 5% drywt/wt basis. A moisture analyzer was used to check the percentage dry weight of the substrate. About 5mg of 5% wt/wt corn stover was pipetted into each serum bottle. Then the serum bottles were autoclaved at 121°C for 45 minutes and left to cool for 1 day. The fourteen inoculum sources were vortexed and then 500µl of each inoculum was pipetted into each serum bottle. Five replicates were made for each inoculum. 1ml of all the isolates from foregut were mixed together and vortexed. 500µl of this was pipetted into 5 serum bottle. This procedure was repeated for the isolates from midgut and hindgut. Also 1ml of all the isolates from all the guts were mixed in a test tube and this consortium was then vortexed and 500µl of it was pipetted into five serum bottles with substrate. The serum bottles were crimped with rubber stoppers. Five serum bottles were kept as blank without adding any inoculum. All the serum bottles with substrates were incubated at 32°C.

The initial weight of the empty serum bottle was measured. Then the weight of serum bottle with inoculum and corn stover was measured and was considered as weight at 0 hrs. The difference between the empty bottle and the bottle with substrate gives the initial basis weight of the substrate. Weight loss readings were noted twice a day. The experiments were run for 120 hrs (5 days). An analytical balance, which could measure up to 4 decimal points with +/- 0.1% accuracy, was used. The serum bottles were vortexed each time the readings were taken.

As a second step the performance of the isolates, which produced weight loss, had to be further assessed, by analytically testing the yields of sugars and ethanol. HPLC (DIONEX, Houston, Texas) was used for this purpose. It was coupled with an RI detector (JASCO, NY). An auto-sampler (Spectra system AS3500) from Thermo Separation Products was used. Peak net release 4.3 software from DIONEX and Ezchromelite software from JASCO were used to interface and run all the equipment. An Ion-exchange Aminex HPX-87H 300 X 7.8 mm (Biorad, Hercules, CA) was used. A guard column (Biorad, Hercules, CA) was attached to the inlet of the column. A Column heater from Chromtech was

used to heat the column and maintain it at 70°C temperature. The temperature of the Refractive Index detector was maintained at 45°C.

The mobile phase used was 0.01N sulfuric acid. Deionized water was added to 278µl of concentrated sulfuric acid to make up total volume to 1L. This solution was filtered using a vacuum filtration system with a 0.47mm dia filter paper. The filtered 0.01N sulfuric acid was degassed with Helium. The flow rate was maintained at 0.6 ml/min.

NREL protocol "Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples", was used for analysis and for generating the calibration curves for glucose, maltose, maltotriose, glycerol, acetic acid and ethanol. The components with their R^2 values, calibration constants, and retention times are shown in table1. A range of concentrations were selected depending on the sensitivity limits of the RI detector. Eight different concentrations were used for each sample. The samples were filtered using a syringe filter assembly and 1ml of each sample was injected into the vial. Then these samples were placed in the auto sampler in ascending order, with concentrations in increasing order. Run time was 35min and sample loop volume was 20µl. The calibration experiments were carried out twice for interday validation.

Further NREL protocol "Lignocellulosic Biomass Hydrolysis and Fermentation LAP-008" will be used to carry out Simultaneous Saccharification and Fermentation (SSF) experiments and determine whether the isolates have had any significant effect on the ethanol yield or not.

Results and Discussion

Out of the fourteen sources isolated and grown only eleven isolates grew successfully. Initially five sources from foregut, five from midgut and four from hindgut were isolated and grown, out of which we could successfully grow four isolates from foregut, four from midgut and three from hindgut. The results of gram staining tests are as follows: Out of the four isolates from foregut, three were found to be gram positive and one gram negative. Two isolates from the midgut were gram positive and two gram negative. Hindgut had two gram positive isolates and one gram negative isolate. Most of the gram-positive isolates are closely related to Bacillus cereus/ Thringiensis.

The pretreated and untreated corn stover showed varying results. The weight loss was observed in the pretreated corn stover while there was hardly any change in the untreated cornstover. Out of the fourteen isolates used only four of them showed weight loss. These four sources were able to reduce the weight of pretreated cornstover by 6%. Essentially these isolates were from the hindgut of the beetle (Three isolates and a consortium of these isolates). In the

gram staining tests it was found that 2 of them are gram positive and one gram negative. In the Biolog tests it was found that the gram positive isolates of hindgut utilized carbohydrates to a large extent and also carboxylic acid and other miscellaneous acids whereas the gram negative isolate utilized carbohydrates and carboxylic acid to an extent and other miscellaneous acids.

Further analysis is required to determine the digested compounds. The reason for only the sources from hindgut being able to reduce the weight could be that different parts of the beetle are at different pHs. The hindgut has a different pH (about 12) when compared to the other parts (Brune and Breznak, 1994). Also different mechanisms take place at different parts, just as in a chemical process. Size reduction is followed by pretreatment step which in turn is followed by enzymatic hydrolysis, fermentation and finally ethanol recovery. Different reactors and different conditions are required at each stage. Similarly in the beetle different conditions are required for the effective working of the digestive track. The weight loss experiments prove that microorganisms do digest lignocellulosic feedstock though the digestion mechanism is yet to be determined. And since there was hardly any change in the untreated cornstover, we could say that pretreatment of biomass is still required to make the cellulose more accessible to the enzymes and to hydrolyze hemicellulose (Carlo N. Hamelinck *et al*., 2003).

As shown in the table below good calibration curves were achieved for all the compounds with good R^2 values. The presence of acetic acid impedes the glucose conversion. So, acetic acid is an important factor which should be evaluated. This is the compound which can be tracked using both UV and RI detectors. By knowing about the concentration of acetic acid before the experiment and after the experiment, measures can be taken so that the acetic acid is separated on time. Due to pretreatment of the cornstover some of the sugars are released and are lost when the feedstock is washed. The amount of glucose and other sugars already present in the cornstover would give us an idea about whether the isolates being used are helping in the saccharification step or not. Finally analyzing for ethanol will show us whether there has been any increase in the ethanol yield. The reason for monitoring glucose, maltose and maltotriose is to check for saccharification of cellulose, as this too is a crucial stage in bioethanol production. . These select analyses will help us analyze the mechanism of each isolate and to design a consortia to produce improved ethanol yield.

Conclusions and Future Studies

Further examination is required to conclude that the isolates have affected the lignocelluloses. We would also analyze cellulose, glucose, other sugars and the end products acetic acid and ethanol to track the changes in them because of the isolates. Also work is under way to determine the characteristics of these

isolates. Our future work aims at performing fermentation experiments to determine the change in ethanol yield with and without the isolates. This will help us determine the efficiency and role of these isolates in increasing the ethanol yield. Also RNA analysis of the isolates is being planned which will help us determine the complete morphology of these inoculum sources. We are also trying to carry out similar experiments using thermophiles. These are the bacteria which can survive in extreme heat conditions.

There exist in nature many insects and microorganisms which feed on lignocellulosic materials. We can investigate them to see whether they are able to digest celluloses and separate them from lignin. Genetic engineering also has to play an important role, as the organisms can be genetically engineered to digest the lignin without affecting the cellulosic components. We can also look at the possibilities of removing acetic acid and other compounds which inhibit the saccharification and fermentation steps and check for increases in the ethanol yield. There is also a need to increase the percentage of solids processed during fermentation for higher efficiency. Currently only 8-10% of the solids are being processed. If this percentage can be increased the efficiency of the ethanol process will also increase.

Table

Component	Concentration Range	RetentionTime (Average)	R^2 Value
Maltotriose	0.28 – 2.78 g/l	8.31 min	0.9971
Maltose	0.35 – 2.77 g/l	8.95 min	0.9940
Glucose	0.3 – 3.0 g/l	10.52 min	0.9995
Glycerol	0.005 – 0.07 M	14.81 min	0.9962
Acetic Acid	0.005 – 0.09 M	16. 29 min	0.9916
Ethanol	0.010 – 0.10 M	22.00 min	0.9975

Table 1 Calibration curves set for analyzing these components and the concentrations used

References

1. Breznak, J. A., and Brune, A. (1994). Role of microorganisms in the digestion of lignocellulose by termites. *Annu. Rev. Entomol*. 39, p.453-87.
2. Carlo N. Hamelinck, Geertji Van Hooijdonk, Andre P.C. Faaij, Prospects for ethanol from biomass: techno-economic performance for development progresses

3. David Galssner (NREL), Jim Hettenhaus (CEA) “Corn Stover approaching its real worth” CTIC meeting, October 27, 1999.
4. Kirk, T.K. and R.L. Farrell. 1987. Enzymatic "combustion": the microbial degradation of lignin. *Annu. Rev. Microbiol*. 41:465-505
5. M.Clark Dale and Mark Moelhman. Enzymatic Simultaneous saccharification and Fermentation (SSF) of Biomass to ethanol in a pilot 130 liter multistage continuous reactor separator
6. Nathan Mosier, Charles Wyman, Bruce Dale, Richard Elander, Y.Y.Lee, Mark Holtzapple, Michael Ladisch, Features of promising technologies for the pretreatment of lignocellulosic biomass, *Biosource Technology 96* (2005) 673-686
7. NREL protocol “Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples“.
8. NREL protocol “Lignocellulosic Biomass Hydrolysis and Fermentation LAP-008”.
9. Ramos, L.P., and Saddler, J.N. (1994) *Appl. Biochem. Biotechnology*. 45/46, p.193-207
10. Sheeshan, J. (2001), the road to bioethanol: A strategic perspective of the U.S. department of energy’s national ethanol program, *American Chemical Society*. P.2-25.
11. Sjostrom, E. (1981), Pulping Chemistry, wood Chemistry: Fundamentals and Applications, Academic Press, 111 Fifth Avenue, New York, NY 1003, P.141.