Evaluating Spent Shiitake and Oyster Mushroom Substrate as Feedstocks for Ethanol Fuel Production

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Summary of Findings

Flower City Mushrooms LLC (FCM) has conducted a pilot scale project to examine the technical and economic feasibility of using spent cellulosic organic mushroom substrate to produce ethanol as an energy product for use on the farm.

Utilizing spent mushroom substrate as a potential feedstock for ethanol fuel production was a consideration sparked by the realization that: (1) increasing demand for low-cost cellulosic materials for biofuel production would very likely increase the cost of mushroom substrate and threaten the marginal economic feasibility of the crop; and (2) the natural biological processes associated with mushroom growth duplicate the thermo-chemical pretreatment and hydrolysis processes associated with current efforts to commercially produce cellulosic ethanol, i.e., to sufficiently degrade lignin so as to increase the availability of cellulose and hemi-cellulose to enzymatic hydrolization to fermentable sugars.

In nature, there are no more efficient biodegraders of lignin than white rot fungi. Shiitake (*Lentinula edodes*), and Oyster (*Pleurotus eryngii* and *Pleurotus sajor caju*) mushrooms both are white rot fungi. These fungi produce laccase enzymes which degrade lignocellulose[1] and, cellulase enzymes, which hydrolyze cellulose to soluble sugars or make them more readily hydrologized than untreated substrate.[2,3]

This work was unique in that it made direct use of the agricultural bi-products that produce lignocellulose decomposing enzymes, rather than attempting to extract those enzymes and applying them to other feedstocks.

Theoretically, as much as 78% of the dry mass of hardwood can be converted to five- and six-carbon sugars[4]. The results of this research reveal that the mycelial growth of white rot fungi did in fact degrade the lignin by approximately 50% in the case of Lentinula edodes on hardwood sawdust substrate and by approximately 25% in the case of Pleurotus sajor caju on wheat straw. Concurrent with lignin reduction, fiber content was reduced by an average of 28% on the sawdust substrate and by 41% on the wheat straw substrate. These reductions in lignin and fiber coincide with a 500% increase in simple sugars from the sawdust substrate and 400% increase in simple sugars from the wheat straw substrate.

While these increases in sugar content may appear significant, they would not be significant enough to result in favorable economic production of ethanol.

- In the case of the sawdust substrate, the 500% increase in simple sugar content represented an increase from only 0.8% (the original sugar content percentage) to approximately 4.8% of the

[1] Sibel S. Kahram and Ismail H. Gurdal, "Effect of synthetic and natural culture media on laccase production by white rot fungi", Bioresource Technology, Volume 82, Issue 3 , May 2002, Pages 215-217
[2] Charles C. Lee ' Dominic W. S. Wong, and George H. Robertson, "Cloning and characterization of two cellulase genes from Lentinula edodes", FEMS microbiology letters (FEMS microbiol. lett.) ISSN 0378-1097 CODEN FMLED7, 2001, vol. 205, n°2, pp. 355-360 (25 ref.), Centre National De La Recherche Scientifique.
[3] "Hydrolysis Fundamentals, US DOE, EERE, Biomass Program, http://www.eere.energy.gov/biomass/hydrolysis_fundamentals.html).
[4] "Understanding Biomass as a Source of Sugars and Energy," US Dept. of Energy, Energy Efficiency and Renewable Energy, 12/13/06, www1.eere.energy.gov/biomass/understanding_biomass.html.

total substrate matter. At this sugar production rate and assuming a 100% efficient fermentation process, only about 41 gallons of fuel ethanol could be produced.

- In the case of the wheat substrate, the 400% increase in simple sugar content represented an increase from only 1.5% (the original sugar content percentage) to approximately 7.5% of the total substrate matter. At this sugar production rate and assuming a 100% efficient fermentation process, only about 64 gallons of fuel ethanol could be produced.

This process and analysis was also performed for *Pleurotus sajor caju* on a corncob substrate. This experiment resulted in the least promising result and the corncob substrate investigation was terminated at this point.

To improve the economic feasibility of fuel ethanol production on spent sawdust and wheat straw substrates, both substrates were subject to enzymatic hydrolysis using two proprietary enzymes made available by Genecor for this research. The hydrolysis process increased simple sugar content by approximately 150% for both substrates when using the Genecor Cellulose Enzyme CP. While addition of an enzymatic hydrolysis step again increased the simple sugar content, the results regarding ethanol production still appear to be economically infeasible. This finding is further supported when the cost of the cellulase enzyme and the process energy costs are considered. The results of this work indicate that conversion of 10,000 pounds of spent mushroom substrate would produce only 51 gallons of fuel ethanol using the shiitake/sawdust substrate formulation, and only 82 gallons of fuel ethanol using the oyster/wheat straw substrate formulation.

The conclusion is that fuel ethanol production from spent mushroom shiitake/sawdust and oyster/wheat straw substrates is economically infeasible.

Methodology

Task 1. Substrate collection.

This work examined the result of mushroom growth on three types of substrate: sawdust, wheat straw, and corncobs. Shiitake mushrooms (Lentinula edodes) were grown on the sawdust substrate and oyster mushrooms (sajor caju) were grown on the wheat straw and corncob substrates. FMC prepared mushroom substrate formulations as planned and collected spent substrate samples at four stages: pre-colonization; post-colonization and pre-fruiting; post-fruiting; and post hydrolysis.

Figure 1 - Wheat straw, corn cob, and saw dust samples prepared for drying and moisture determination.

15 samples were collected and labeled as follows:

- sd1: pre-colonization sawdust
- sd2: post-colonization and pre-fruiting sawdust
- sd3 & sd4: post-fruiting sawdust
- phsd3a, phsd3b & phsdbl: post-hydrolysis sawdust
- ws1: pre-colonization wheat straw
- ws2: post-colonization and pre-fruiting wheat straw
- ws3: post-fruiting wheat straw
- phws3a & phws3b: post-hydrolysis wheat straw
- cc1: pre-colonization corncob
- cc2: post-colonization corncob
- cc3: post-fruiting colonization corncob

Task 2. Pre-Process Sample Preparation and Analyses

1. Collected substrates were sampled and prepared for analysis following NREL Laboratory Analytical Procedure (LAP) 2004, Preparation of Samples for Compositional Analysis http://devafdc.nrel.gov/pdfs/9362.pdf).

Figure 2 - Drying samples.

Figure 3 – Dried Sample.

Figure 3-- Grinding sample.

Figure 5 - Coarse screen.

Figure 6 - Fine screen.

Figure 7 - Dried and screened sample.

Samples were dried using Preparation Method B: Convection oven drying. This method is suitable for very wet biomass that is at risk for microbial growth during drying, wet pretreated biomass, samples that would not be stable during prolonged exposure to ambient conditions, or for drying materials when ambient humidity does not allow the sample to air-dry to a moisture content below 10% as measured using LAP "Determination of Total Solids in Biomass" (2004). This drying method is suitable for small samples of biomass (<20 g).

- 10.3.1 Select a container suitable for oven drying the biomass sample and dry this container at 45± 3°C for a minimum of 3 h.
- 10.3.2 Place the container in a dessicator and allow the container to cool to room temperature.
- 10.3.3 Weigh the container to the nearest 0.1 g and record this weight as Wt.
- 10.3.4 Place the biomass material into the dried container to a maximum depth of 1 cm.
- 10.3.5 Weigh the container and biomass to the nearest 0.1 g and record this weight as Wi.
- 10.3.6 Place the container and biomass in a drying oven maintaining the temperature at 45 ± 3°C. Allow the material to dry for 24 to 48 h.
- 10.3.7 Remove the container and biomass from the drying oven, place in a desiccator and allow the sample to cool to room temperature.
- 10.3.8 Weigh the container and biomass to the nearest 0.1 g and record this weight as Wf.

Project Problem and Resolution: Prepared samples were to be analyzed without cost by the National Renewable Energy Laboratory (NREL) using NREL LAP 009, Enzymatic Saccharification of Lignocellulosic Biomass (http://devafdc.nrel.gov/pdfs/9578.pdf) to determine the extent to which fermentation was possible (essentially an assay of simple and complex sugars). However, the NREL staff person who was originally contacted at the NREL regarding this work during the proposal development phase was no longer employed there when it came time to perform the analyses and the current director informed the project team that he would have to charge $3,000 per sample for this service. Since there were at least nine sample that needed to be analyzed, this cost was well beyond the project's budgetary capabilities.

The loss of this promised service was a major setback to the project. Apparently only the National Renewable Energy Laboratory is equipped to conduct this rigorous analysis and project staff spent many days contacting private labs in attempting to remedy this unfortunate situation. Two alternative strategies were employed to provide a solution.

- The first was to analyze the samples to compare fiber content and water soluble carbohydrates before inoculation, after mycelial growth, and after fruiting. This methodology could provide evidence of fiber hydrolization and is described further in Task 3.1 below.

- The second was recommended by Dr. Michael Haselkorn from Rochester Institute of Technology's Center for Integrated Manufacturing Studies (RITCIMS) as an alternative strategy for determining whether the mycelial growth phase of mushroom production increased the extent to which the lignocellulose materials would be hydrolysable. RIT is involved in similar work evaluating silage as a potential feedstock for ethanol production. Dr. Haselkorn's methodology is described in Task 3.2.

Both methodologies were used.

Task 3. Process sequence and logistics

1. **Comparison of fiber content and water soluble carbohydrates.**

- Methodology
  - Ten samples were sent to Dairy One Forage Testing Laboratory in Ithaca, NY for analysis of:
    - Moisture
    - Dry Matter
    - Acid Detergent Fiber (ADF)
    - Neutral Detergent Fiber (NDF)
    - Lignin
    - Water Soluble Carbohydrates (WSC)
  - Lignin, Cellulose, Hemicellulose and Total Fiber were calculated from the results using the following equations:
    - Cellulose = ADF – Lignin
    - Hemicellulose = NDF – ADF
    - Total Fiber = Cellulose + Hemicellulose

- Analysis results were evaluated to determine whether or not additional enzymatic hydrolysis is necessary to increase bioavailability of cellulosic sugars;
  - Focusing principally on the loss of total fiber and formation of simple sugars as an indicator of hydrolysis, these results indicate that some hydrolysis occurred

in each case from the point of inoculation through vegetative growth and fruiting but that additional hydrolysis would be necessary to achieve adequate concentrations of cellulosic sugars to effectively ferment them into ethanol.

- To accomplish this, two different cellulase enzymes were procured from the Genecor Corporation (www.Genecor.com):
  - Genecor Cellulase Enzyme GC 220; and
  - Genecor Cellulase Enzyme CP

2. **Haselkorn Methodology – Part 1: Visual (microscopic) Evaluation**
Following the advice of Dr. Haselkorn, samples were visually inspected for evidence of lignin destruction.

Haselkorn Methodology – Part 2: Determine changes in mass.
Again, following the advice of Dr. Haselkorn, pre-inoculation and post-fruiting substrate samples were taken, dried, weighed and then using the Genecor cellulase enzyme, hydrolyzed over a 24 hour period. The samples were then screened, dried and weighed again to determine the percentage of the solids hydrolyzed.

Figure 8 - Microscopic assessment of lignin deterioration

Fig. 9

Fig. 10

Fig. 11

Fig. 12

Microscopic examination of pre-colonization and post-fruiting substrates provided some evidence of cell wall breakdown.

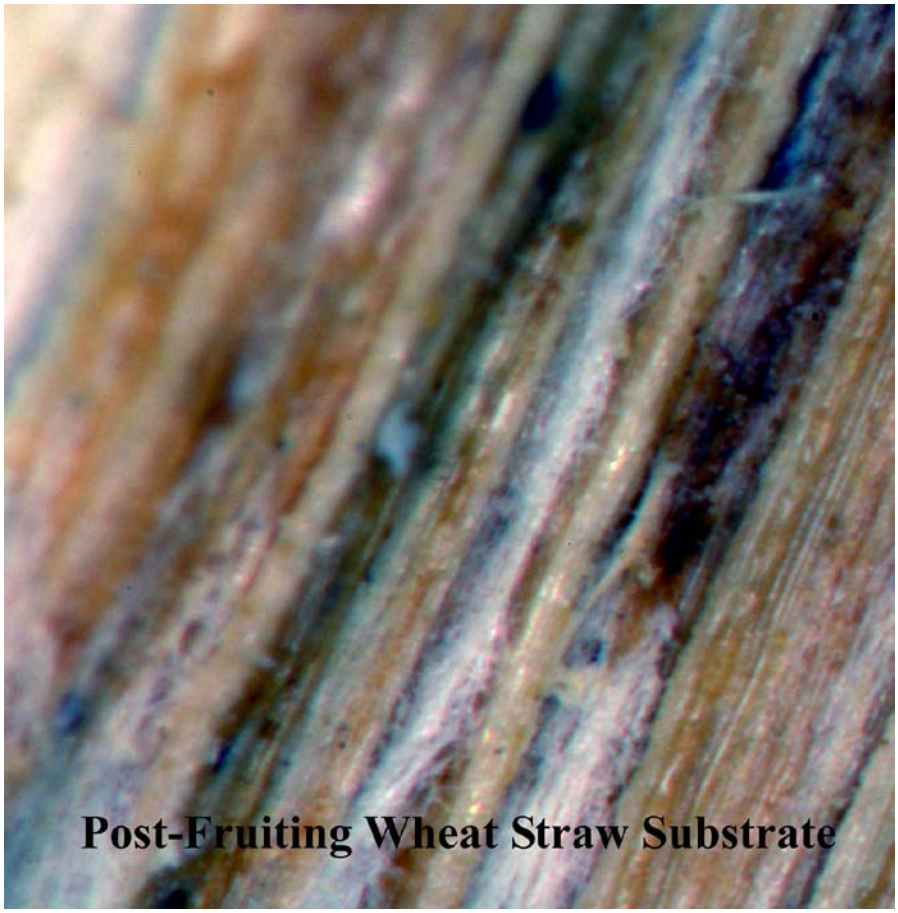

Fig. 13

Figure 14 – Grinding sample for hydrolysis

Figure 15 – Hydrolysis set up

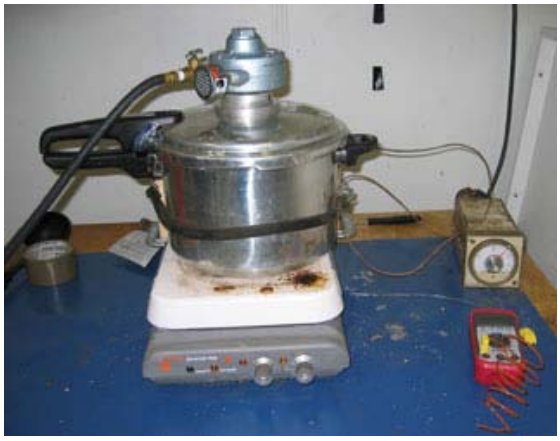

Figure 16 – Hydrolysis set up

Figure 17 – Putting post-hydrolysis sample in filter press

Figure 18 – Extract removal

Figure 90 – Post-hydrolysis extract samples

Task 4. **Fermentation Tank Design Selection and Construction** A fermentation tank was purchased for this work but not used due to the low yield of fermentable sugars after mycelial growth followed by enzymatic sacchrification.

Task 5. **Simultaneous Sacchrification and Fermentation**

FCM did not run simultaneous sacchrification and fermentation steps. Enzymatic sacchrification was performed using the RITCIMS laboratory.

Task 6. **Ethanol Still Design Selection and Construction**

A small distillation still was purchased and set up for this work. However, since the fermentation step was eliminated, the need for distillation was obviated.

Task 7. **Distillation**

In preparation for the production of ethanol alcohol, a regulated substance, FCM applied for and received an Alcohol Fuel Producer Permit, pursuant to Part 5181 of Title 26 of the US Code, from the Department of the Treasury Alcohol, Tobacco Tax and Trade Bureau (See Appendix K). However, d to the relatively low sugar production rates achieved, the fermentation and distillation steps were not warranted.

Task 8. **Economic Analysis & Enterprise Budget.**

Research results indicate that fuel ethanol production from spent mushroom substrate is not economically feasible and thus no enterprise budget is necessary.

Task 9. **Business Plan.**

Lack of economic feasibility obviated the need for this task.

Task 10. **Final Report.**

Submitted February 23, 2009.

Task 11. **Information Dissemination**

This report has been submitted to:

The Mushroom Growers' Newsletter
P.O. Box 5065
Klamath Falls, OR 97601 USA

Analysis and Discussion

Pre-Hydrolysis Sawdust/Shiitake Substrate Analyses *(See Appendices A & D)*

Fiber Analysis

Hemicellulose

Hemicellulose degradation was the most dramatic of changes to fiber content. Hemicellulose content was reduced by 20% during the vegetative, mycelial growth phase and reduced by an additional 67% during the fruiting phase to reduce the original hemicellulose in this substrate by approximately 87% of its original content. It is indicated by these findings that hemicellulose is readily degraded by Lentinula edodes and that the resulting products of that degradation are utilized principally in fruiting of the mushrooms.

Lignin

Lignin was degraded dramatically as a result of mycelial growth during the pre-hydrolysis stages of this work. The most dramatic change occurred during the vegetative growth phase, when lignin content was reduced by 37.27%. Lignin content was reduced an additional 13 to 14% during the fruiting phase. It is indicated by these findings that lignin is also readily degraded by Lentinula edodes. While it may also appear that the resulting products of that degradation are utilized principally in the vegetative growth of the mycelia, the literature indicates that white rot fungi can convert lignin completely to carbon dioxide.[5]

Cellulose

Of the three fiber components in this substrate, cellulose content was reduced the least. Cellulose content was reduced by only approximately 10% across both vegetative mycelial growth and fruiting phases. There was some inconsistency in the analytical results in that total cellulose content reduction after the fruiting phase was not (though it should have been) greater than that after the mycelial growth phase.

Fiber Analysis Summary

Total fiber content was reduced by approximately 14% in the mycelial growth phase and reduced further by another 14% during the fruiting phase, bringing the total reduction to approximately 28%. If lignin degradation is discounted as a contributor to mushroom growth (in both vegetative and fruiting phases) due to the likelihood that much of it was converted to carbon dioxide, then we might focus on hemicellulose and cellulose as the principal carbon sources for both sugar

[5] **"The Fungus Among Us,"** *Environmental Health Perspectives* Volume 101, Number 3, August 1993**, accessed online, Feb. 15, 2009.**

production and mushroom growth. Of the total substrate material, 72% is represented by the combined content of cellulose (52.9%) and hemicellulose (19.2%). A reduction of 87% of the hemicellulose content therefore represents a reduction of (87% x 19.2%=) 16.7% of the total substrate material, and a reduction of approximately 10% of the cellulose content represents a reduction of (10% x 52.9%=) 5.29% of the total substrate material.

Pre-Hydrolysis Wheat Straw/Oyster Substrate Analyses *(See Appendices A & E)*

Fiber Analysis

Hemicellulose

Hemicellulose degradation was again the most dramatic of changes to fiber content in the wheat straw/oyster substrate samples. Hemicellulose content was reduced by 59.53% during the vegetative, mycelial growth phase and reduced by an additional 29.19% during the fruiting phase to reduce the original hemicellulose in this substrate by approximately 89% of its original content, very similar to the sawdust/shiitake findings. It is indicated by these findings that hemicellulose is readily degraded by Sajor caju and that the resulting products of that degradation are utilized principally in the vegetative growth phase of mushroom production.

Lignin

Lignin was degraded less dramatically than was hemicellulose. While the analytical results are somewhat inconsistent for these samples, it appears that the greatest reduction occurred during the vegetative growth phase, when lignin content was reduced by approximately 29%. Since the results of the post-fruiting lignin content was actually less (21%) than this, it does not appear that additional reduction occurred during the fruiting phase. It is indicated by these findings that lignin is also readily degraded by Sajor caju. Again, while it may also appear that the resulting products of that degradation are utilized principally in the vegetative growth of the mycelia, the literature indicates that white rot fungi can convert lignin completely to carbon dioxide.[6]

Cellulose

Again, cellulose content was reduced the least of the three fiber components. Cellulose content was reduced by only approximately 12% across both vegetative mycelial growth and fruiting phases. There was also some inconsistency in the analytical results in that total cellulose content reduction after the fruiting phase was not (though it should have been) greater than that after the vegetative mycelial growth phase. It does appear that the reduction occurred principally during the vegetative mycelial growth phase.

[6] **"The Fungus Among Us,"** *Environmental Health Perspectives* Volume 101, Number 3, August 1993**, accessed online, Feb. 15, 2009.**

Fiber Analysis Summary

Total fiber content was reduced by approximately 15% in the mycelial growth phase and reduced further by another 26% during the fruiting phase, bringing the total reduction to approximately 41%. Again, if lignin degradation is discounted as a contributor to mushroom growth (in both vegetative and fruiting phases) due to the likelihood that much of it was converted to carbon dioxide, then we might focus on hemicellulose and cellulose as the principal carbon sources for both sugar production and mushroom growth. Of the total substrate material, 66.4% is represented by the combined content of cellulose (40.7%) and hemicellulose (25.7%). A reduction of 89% of the hemicellulose content therefore represents a reduction of (89% x 25.7% =) 22.9% of the total substrate material, and a reduction of approximately 12% of the cellulose content represents a reduction of (12% x 40.7%=) 4.88% of the total substrate material.

Sugar Analysis *(See Appendices A & C)*

All samples were analyzed for water soluble sugar content.

Shiitake/Sawdust Samples

Sugar content increased most dramatically during the vegetative mycelial growth phase, increasing by approximately 460%. Sugar content increased by another approximate 40% during the fruiting stage bringing the total increase to 500%, i.e., five times their original sugar content. This represents an increase from 0.8% of the original substrate material to a final content of 4.8%.

Again, the principal portion of this fivefold increase occurred during the vegetative mycelial growth phase and this appears to correspond more closely with the cellulose reductions, then with a hemicellulose reductions. Both the cellulose reduction and the sugar increase during the vegetative mycelial growth phase represent an approximate change of 5% of the total substrate content, and we might thus expect that out of 100 pound sample of substrate, 5 lb. of cellulose fiber would be converted during shiitake mushroom production to 5 lb. of water soluble sugar.

Oyster/Wheat Straw Samples

Sugar content increased most dramatically during the vegetative mycelial growth phase, increasing by approximately 340%. Sugar content increased by another approximate 60% during the fruiting stage bringing the total increase to 400%, i.e., four times the original sugar content. This represents an increase from 1.5% of the original substrate material to a final content of 7.5%.

Again, the principal portion of this fivefold increase occurred during the vegetative mycelial growth phase and this appears to correspond with both the cellulose and hemicellulose reductions. The reductions in cellulose and hemicellulose during this phase represent an approximate change of (13.76% x 40.7 =) 5.6% for the cellulose plus (59.53% x 25.7% =)

15.29% for the hemicellulose for a total reduction of approximately 21% of the substrate content and we might thus expect that out of 100 pound sample of substrate, approximately 21 lb. of cellulose and hemicellulose fiber would be converted during oyster mushroom, producing approximately 7 lb. of water soluble sugar. While this is not as neat as the one-to-one correspondence demonstrated with the shiitake/sawdust samples, it still reasonably indicates a correlation between the simultaneous reduction in fiber and increase in sugar.

Post-Hydrolysis Analysis *(See Appendices G, H I & J)*

The hydrolysis process increased simple sugar content by approximately 150% for both substrates when using the Genecor Cellulose Enzyme CP. While addition of an enzymatic hydrolysis step again increased the simple sugar content, the results regarding ethanol production still appear to be economically infeasible. This finding is further supported when the cost of the cellulase enzyme and the process energy costs are considered.

- In the case of the sawdust substrate, the total (500 + 154=) 654% increase in simple sugar content represented an increase to approximately 6.04% of the total substrate matter. At this sugar production rate and assuming a 100% efficient fermentation process, only about 51 gallons of fuel ethanol could be produced.
- In the case of the wheat substrate, the total (400 + 141 =) 541% increase in simple sugar content represented an increase to approximately 9.62% of the total substrate matter. At this sugar production rate and assuming a 100% efficient fermentation process, only about 82 gallons of fuel ethanol could be produced.

Ethanol Production Calculations *(See Appendix C)*

Since the amount of sugar potentially available from the spent substrate was so low as to make production economically infeasible, the fermentation process was not conducted and potential ethanol production was projected using the standard conversion of: 1 lb. sugar ⇨ 0.5 lb. ethanol, at 100% conversion efficiency.

Conclusions

The loss of analytical services originally thought to have been committed by the National Renewable Energy Laboratory resulted in limited comprehension regarding the breakdown products during the various steps in the conversion process. However, alternative procedures were developed and carefully followed and these findings are presented with confidence.

This work provides evidence that the use of spent shiitake/sawdust and oyster/wheat straw substrate formulations as feedstock for fuel ethanol production is economically infeasible.