Nicole Buyck attended Colorado State University majoring in Education and Biology. Prior to her appointment at NREL, she volunteered for the Rocky Mountain Raptor Program as well as provided environmental education through the Sierra Club Alliance at CSU. Nicole aspires to be a high school biology teacher. She wants to help bring out the hidden talents of those she works with in the classroom setting. Her research at NREL, looking at the cell wall variation of Zea mays *mutants, gave her a better understanding of scientific research. She hopes to bring this understanding to her future students.*

Steven R. Thomas is a Senior Scientist at the National Renewable Energy Laboratory, where he has been employed since 1992. His interests in the area of biomass conversion include characterization of cellulase enzymes and their genes, genetic improvement of cellulases, and genetic engineering of crop plants to produce inexpensive cellulase enzymes for use in biomass conversion. During the last two years, Dr. Thomas has headed a project to screen a set of mutant lines of corn to find mutations that affect cell wall composition. Dr. Thomas holds a Ph.D. in Biology from UCLA and received his Bachelor's degree in Biology from UC San Diego.

DETECTION OF CELL WALL CHEMICAL VARIATION IN *ZEA MAYS* MUTANTS USING NEAR-INFRARED SPECTROSCOPY

NICOLE BUYCK AND STEVEN THOMAS PH.D.

ABSTRACT

Corn stover is regarded as the prime candidate feedstock material for commercial biomass conversion in the United States. Variations in chemical composition of *Zea mays* cell walls can affect biomass conversion process yields and economics. Mutant lines were constructed by activating a Mu transposon system. The cell wall chemical composition of 48 mutant families was characterized using near-infrared (NIR) spectroscopy. NIR data were analyzed using a multivariate statistical analysis technique called Principal Component Analysis (PCA). PCA of the NIR data from 349 maize leaf samples reveals 57 individuals as outliers on one or more of six Principal Components (PCs) at the 95% confidence interval. Of these, 19 individuals from 16 families are outliers on either PC3 (9% of the variation) or PC6 (1% of the variation), the two PCs that contain information about cell wall polymers. Those individuals for which altered cell wall chemistry is confirmed with wet chemical analysis will then be subjected to fermentation analysis to determine whether or not biomass conversion process kinetics, yields and/or economics are significantly affected. Those mutants that provide indications for a decrease in process cost will be pursued further to identify the gene(s) responsible for the observed changes in cell wall composition and associated changes in process economics. These genes will eventually be incorporated into maize breeding programs directed at the development of a truly dual use crop.

INTRODUCTION

Corn stover (the stalks and leaves of *Zea mays*) has been identified by the Department of Energy's Biofuels Program as the prime candidate feedstock material for commercial biomass conversion in the United States. Although environmental changes can influence genetic programming, a large component of the variability in the chemical composition of corn stover can be explained by the genetics of the individual. For example, previous work with brown midrib corn (*Z. mays*) varieties indicates lower lignin composition (Barrière and Argillier, 1994). Variations in chemical composition of plant cell walls can affect biomass conversion process yields and economics. For instance, as the proportion of lignin in plant cell walls rises, the proportion of some other component must decrease. If polysaccharide content decreases, the overall ethanol yield per ton of input biomass decreases. As ethanol yield decreases, sales of product also decline, eventually to the point of non-profitability. This study advances our understanding of how genetic variability in cell wall composition of *Zea mays* affects biomass conversion.

Previous efforts by scientists at the National Renewable Energy Lab to screen a portion of a mutant library of corn yielded 106 candidate mutant pools from a collection of approximately 5000 individuals screened. The 5000 individuals represented 244 pools of 12 mutant families each (2928 mutant families). Mutant lines were constructed through the efforts of others (see URL http://mtm.cshl.org) by activating a Mu transposon system that causes the Mu genetic element to move from one location in a chromosome and re-insert itself into another location (transposition) (Alleman and Freeling, 1986). Of the 106 candidates, four pools of mutants were deconvoluted and re-grown as 48 separate families during the winter of 1999-2000. A total of 349 mature, green plants were harvested from these families and shipped to the National Renewable Energy Lab (NREL) in Golden, Colorado, for further analysis. Characterization of these plants using near-infrared (NIR) spectroscopy is the subject of this study.

Screening of maize plants from these 48 mutant families with NIR spectroscopy and multivariate statistical techniques has identified 57 individuals as chemically unusual in one or more of the six Principal Components produced in the analysis. The individuals were identified as unusual relative to a normally distributed population at the 95% confidence level. Of these, 19 individuals

from 16 families are identified by the two PCs that describe variance in cell wall polymers. These 16 families will be subjected to further analysis.

MATERIALS AND METHODS

Mutant plants screened in this study were kindly provided by Dr. Erik Vollbrecht at the Cold Spring Harbor Laboratory in Cold Spring Harbor, NY. *Zea mays* plants were grown at Cold Spring Harbor Laboratory's winter nursery in Hawaii using standard farming methods during the winter of 1999-2000. Twelve sibling seeds from 48 genetically segregating F2 mutant maize families, belonging to four previously identified chemical outlier mutant pools, were planted and grown to maturity. Above ground portions of whole plants were harvested at maturity while still green and stored in breathable polypropylene onion bags with a paper tag identifying the family. Plants were shipped via overnight express to the NREL and upon arrival the plants were dried at 50 °C for 48 hours with vigorous ventilation of the chamber. Dried plants were stored at room temperature for approximately 2 months before processing for NIR spectroscopy.

Each of the mutant lines is genetically segregating for each transposon insertion event in the genome (~200 Mu insertions per genome). Mutations caused by Mu insertions usually give rise to recessive Mendelian phenotypes, therefore only one quarter of the individuals in a segregating F2 family are expected to display a given mutant phenotype. Thus, detection of multiple individuals per family is preferred in order to confirm mutant phenotypes, which in this case constitutes an altered chemical composition of leaf tissue. Because we obtained several individuals per family, each plant was assigned a unique identification number, corresponding to the Hawaii mutant code and the plant number from that family, numbered consecutively from 1 (e.g., 764-1).

The leaves from each individual were harvested, excluding the flag leaves located just below the tassel and the juvenile leaves located at the base of the stalk. Only the leaf blades (from the distal leaf tip to the ligule, including the midrib) were harvested and uniquely labeled. The leaf sheath was not harvested.

In a previous study at NREL (unpublished results), it was determined that data collection is best performed after the leaf tissue has been coarsely ground. Thus, each bag of leaves was fragmented using a hand-held kitchen blender (Braun, models MR430HC and MR370). Although particle size varied, (ranging from a fine powder to 10 millimeters in diameter) the average dimension was approximately 2 millimeters.

NIR spectroscopy (FieldSpec Pro, model No. FSP350-2500P; Analytical Spectra Devices, Inc., Boulder, CO) data were collected for the ground maize leaves with the fiber optic probe located 4.5 cm above the sample, yielding a circular 2 cm diameter field of view. A white light source was located 36 cm above the samples, at an angle of 30 degrees from vertical. Leaf samples were placed in an ordinary soda bottle-cap and placed beneath the probe for analysis. Thirty spectra were collected from each sample and averaged for use in statistical analysis of the results.

Data were analyzed using a multivariate statistical analysis technique called Principal Component Analysis (PCA) (Meglen, 1992). PCA was accomplished using computer software entitled "The Unscrambler" (by Camo Software, P.C. version 7.5).

RESULTS

PCA of the NIR data from 349 maize leaf samples reveals 57 individuals as chemically unusual on one or more of six Principal Components (PCs) at the 95% confidence interval. Of these, 19 individuals from 16 families score as non-normal on either PC3 or PC6, the two PCs that contain information about cell wall polymers. PC1 and PC2 explained 68% and 18% of the total variance among the samples, respectively. The PC-loading suggests that PC1 accounts for differences in the brown color of the samples (Figure 1). We suspect that this is largely because of the degree to which the plants were hosting a surface fungal colonization. PC2 explains differences in leaf thickness, which causes variation in signal intensity among the samples (Figure 2). These components are not pertinent to the chemical composition of the leaves and will not be discussed further.

The remaining four PCs contain chemical information that may be of value from the point of view of biomass conversion. PC3 explains 9% of the variation among all the samples and the PC-loading suggests this variation is due to a combination of chlorophyll and cellulose content (Figure 3). PC4 explains 3% of the variance among all the samples and the PC-loading contains no information about cell wall polymers (Figure 4). The PC-loading for PC5 suggests that 1% of the variance can be explained by the chlorophyll and carotenoid content of the samples and carries

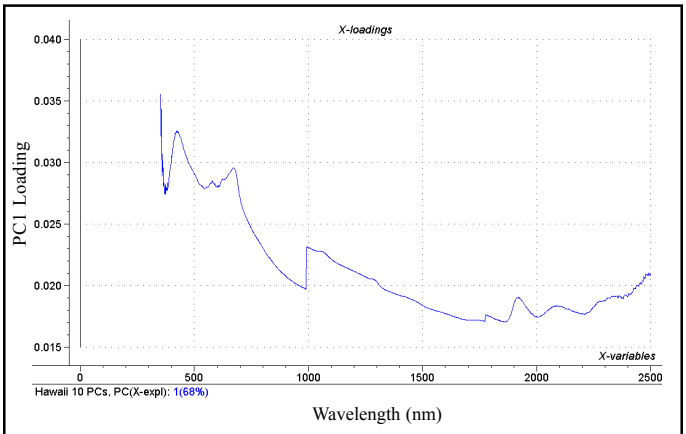

Figure 1. Principal Component 1 Loading Profile

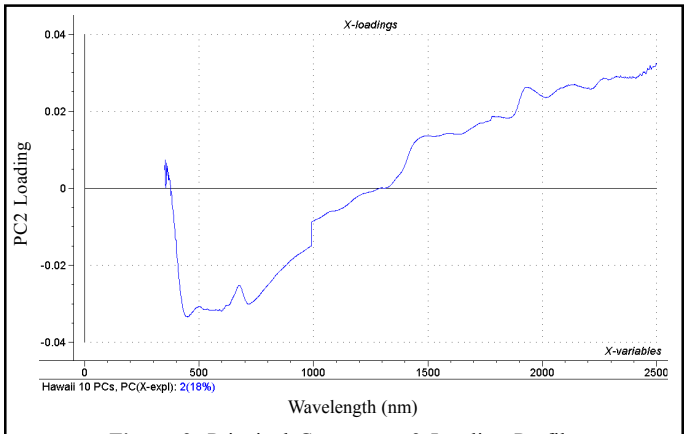

Figure 2. Principal Component 2 Loading Profile

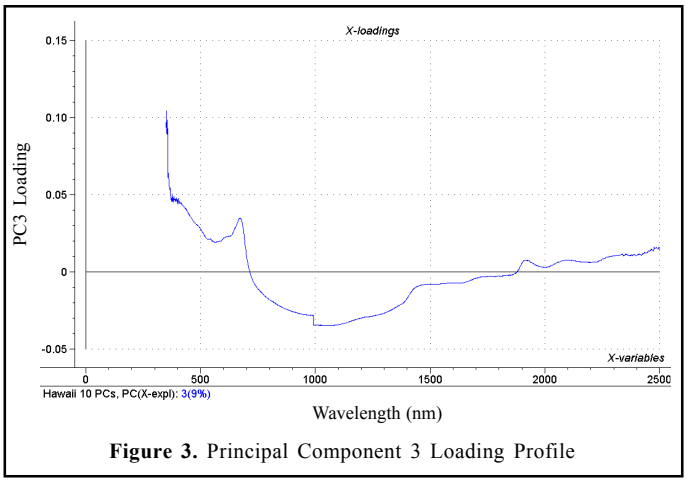

Figure 3. Principal Component 3 Loading Profile

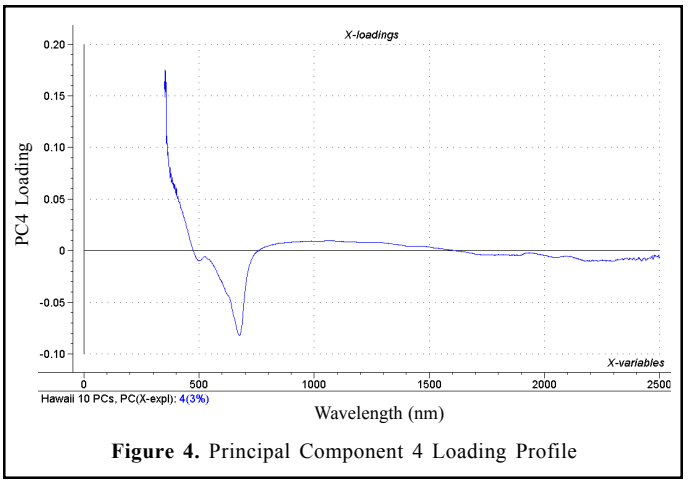

Figure 4. Principal Component 4 Loading Profile

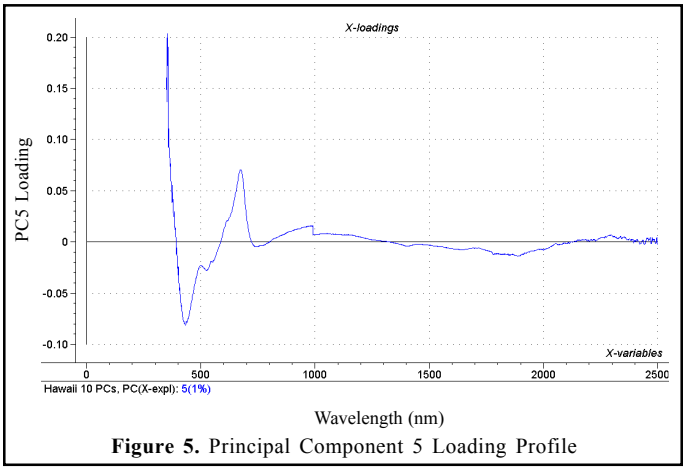

Figure 5. Principal Component 5 Loading Profile

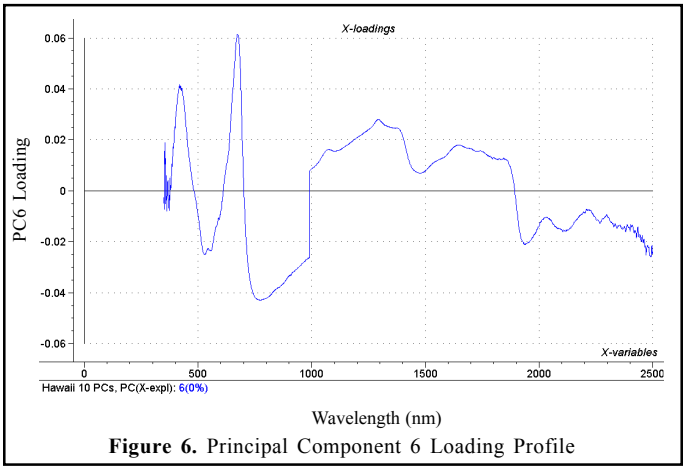

Figure 6. Principal Component 6 Loading Profile

no information about cell wall polymers (Figure 5). Lastly, PC6 explains less than 1% of the variance among all the samples and the PC-loading plot suggests this is due to a combination of chlorophyll, carotenoid, and sugar content (Figure 6). Chlorophyll (green) and carotenoids (yellow, orange and red) are common leaf pigments visible to the human eye.

Of the six Principal Components, PC3 and PC6 contain information relevant to plant cell wall polymers important for biomass conversion. Scores for PC3 and PC6 were plotted for each individual sample (PC3 versus PC6), and the plot identifies 22 unusual spectra at the 95% confidence level (Figure 7).

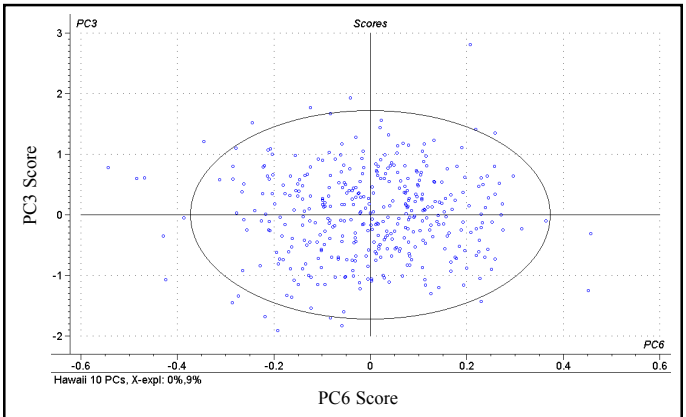

Figure 7. Plot of Principal Component Scores for PC3 Versus PC6 There is only a 5% chance that points outside the black oval belong to a normally distributed population that is described by the points lying inside the oval. Points lying outside the oval probably have unusual cell wall chemistry.

DISCUSSION

The 22 unusual spectra identified in Figure 7 correspond to 19 individuals from 16 families. These 22 individuals are listed in Table 1. Three of these are replicate scans of the same sample (633-4) and two others are replicate scans from a different family (623-9). In Table 1 there are three cases where two different individuals from the same family (boldface type) are listed as outliers. These three families (624, 758 and 762) are important because the presence of two outlier individuals in the same family lends greater credence to the hypothesis that the family has unusual cell wall chemistry caused by a genetic mutation. In particular, it should be noted that family 624 shows 2 outliers out of 7 total plants, family 758 shows 2 outliers out of 9 total plants, and family 762 shows 2 outliers out of 7 total plants screened. In each case, the number of unusual individuals approximates one quarter of the individuals screened from each family, lending further credence to the genetic source of the variability detected. These three families will be the prime focus of future studies emanating from this work, although all 16 families will be considered for further analysis.

Future studies will involve quantitative wet chemical analysis to confirm the NIR results on the outlier individuals. Those

individuals for which altered cell wall chemistry is confirmed will then be subjected to fermentation analysis to determine whether or not biomass conversion process kinetics, yields and/or economics are significantly affected. Those mutants that provide indications for a decrease in process cost will be pursued further to identify the gene(s) responsible for the observed changes in cell wall composition and associated changes in process economics. These genes will eventually be incorporated into corn breeding programs directed at the development of a truly dual use crop.

ACKNOWLEDGEMENTS

Special thanks to Steven Thomas, my mentor scientist, who provided me with the opportunity to work on this project and offered continual support throughout my experience at the National Renewable Energy Laboratory. Special thanks, also, to Robert Meglen for helping me with the data collection and analysis. I would also like to thank Cheryl Jurich and Renee Lagutaris for their assistance in the laboratory.

My gratitude is also extended to the Pre-Service Teacher (PST) Program Coordinator, Robi Robichaud and the Master Teacher for the program, John Sepich. In addition I would like to thank Todd Burke (teacher mentor) for his continual support.

Without the support from the Department of Energy, NREL, the National Science Foundation, and the Rocky Mountain Teacher Education Collaborative, this research opportunity would not have been possible. Thanks to the many people from these organizations for putting together a wonderful program.

Table 1. Spectra Scores for Components 3 and 5
Individual spectra that that score outside the 95% confidence interval for a normally distributed population for principal components 3 and 5.

Hawaii Family #	Individual #	CSHL Family #	Spectrometer Filename
606	10	3668	H2.169
609	10	3671	H2.168
623	9	3961	H2.042
623	9	3961	H2.003
624	**1**	**3962**	**H2.123**
624	**3**	**3962**	**H1.013**
629	2	3967	H2.072
633	4	3971	H1.138
633	4	3971	H1.110
633	4	3971	H1.126
634	5	3972	H1.176
750	3	9170	H1.116
755	5	9176	H2.099
756	1	9177	H2.110
758	**4**	**9179**	**H1.124**
758	**8**	**9179**	**H1.141**
759	3	9180	H2.089
761	1	9602	H2.158
762	**6**	**9603**	**H2.154**
762	**7**	**9603**	**H1.002**
764	5	9606	H2.164
768	6	9610	H2.066

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Alleman, M., Freeling, M. (1986). "The Mu transposable elements of maize: evidence for transposition and copy number regulation during development." Genetics . 112, pp. 107-19.

Barrière, Y., Argillier, O. (1994). "Brown-midrib genes of maize: a review." Agronomie. 13, pp. 865-876.

Meglen, Robert R. (1992). "Examining large databases: a chemometric approach using principal component analysis." Marine Chemistry. 39, pp. 217-237.

EDUCATION MODULE

TITLE

A Scientific Investigation on Alcohol Fermentation and Biomass Conversion

AUTHOR

Nicole Buyck

GRADE LEVEL/SUBJECT

AP (Advanced Placement) Biology
Grades 11 and 12
90 minute periods

CURRICULUM STANDARD: AAAS BENCHMARKS. PROJECT 2061.

Section 1, The Nature of Science.

By the end of the 12th grade, students should know:

- **1B Scientific Inquiry:** Hypotheses are widely used in science for choosing what data to pay attention to and what additional data to seek, and for guiding the interpretation of the data (both new and previously available).

Section 5, The Living Environment.

By the end of 12th grade, students should know:

- **5C Cells:** Complex interactions among the different kinds of molecules in the cell cause distinct cycles of activities, such as growth and division. Cell behavior can also be affected by molecules from other parts of the organism or even other organisms.

Section 9, The Mathematical World.

By the end of 12th grade, students should know:

- **9B Symbolic Relationships:** In some cases, the more of something there is, the more rapidly it may change (as the number of births is proportional to the size of the population). In other cases, the rate of change of something depends on how much there is of something else (as the rate of change of speed is proportional to the amount of force acting).

- **9B Symbolic Relationships:** Tables, graphs, and symbols are alternative ways of representing data and relationships that can be translated from one to another.

Section 11, Common Themes.

By the end of 12th grade, students should know that:

- **Constancy and Change:** Graphs and equations are useful (and often equivalent) ways for depicting and analyzing patterns of change.

Section 12, Habits of Mind.

By the end of 12th grade, students should be able to:

- **12B Computation and Estimation:** Use computer spreadsheet, graphing, and database programs to assist in quantitative analysis.
- **12B Computation and Estimation:** Compare data for two groups by representing their averages and spreads graphically.
- **12C Manipulation and Observation:** Learn quickly the proper use of new instruments by following instructions in manuals or by taking instructions from an experienced user.
- **12C Manipulation and Observation:** Use computer technology for producing tables and graphs and for making spreadsheet calculations.
- **12D Communication Skills:** Choose appropriate summary statistics to describe group differences, always indicating the spread of data as well as the data's central tendencies.
- **12D Communication Skills:** Participate in group discussions on scientific topics by restating or summarizing accurately what others have said, asking for clarification or elaboration, and expressing alternative positions.
- **12D Communication Skills:** Use tables, charts, and graphs in making arguments and claims in oral and written presentations.

OVERVIEW

This three-day lesson will allow students to enhance their laboratory technique, as well as familiarize them with using software (such as Excel) to manipulate data, create graphs, and interpret results. Students will perform two investigations concerning biomass conversion to ethanol. Students are expected to use the scientific method in order to create their own scientific investigation.

PURPOSE

The purpose of this lesson is three-fold:

- To introduce students to the industrial uses of metabolic pathways, especially biomass conversion using fermentation.
- To encourage students to use the scientific method in order to create their own scientific investigation.
- To enhance laboratory technique and introduce data analysis using computer software (such as Microsoft Excel).

LEARNING OBJECTIVES

- Students will be introduced to the industrial uses of metabolic pathways.
- Students will gain a better understanding of the fermentation process and its industrial use for biomass conversion.
- Students will be able to use the scientific method to create their own scientific investigations.
- Students will be able to use computer software (Microsoft Excel) to create spreadsheets for data, as well as graphs (including appropriate equations and statistical calculations).
- Students will be able to correctly interpret data, and to use charts and graphs to communicate their findings to others.
- Students will be able to compare data sets and draw educated conclusions about causes of variation.

VOCABULARY

Amylase
Assay
Buffer
Cuvette
Ethanol
Fructose
Metabolic Pathway
Non-Renewable Energy
Spectrometer
Starch
Yeast Media
Anaerobic
Biomass
Centrifuge
Enzyme
Fermentation
Glucose
Microsoft Excel
Renewable Energy
Spreadsheet
Sucrose

MATERIALS

Fermentation Materials

- **Safety glasses**
- Cornstarch (or soluble potato starch)
- Table sugar (sucrose)
- Fructose
- Glucose (dextrose)
- Peptone
- Yeast extract
- Baker's yeast
- Other yeast varieties
- Amylase enzymes:
  - Maxamly from Gist-brocades
  - Amyloglucosidase from Sigma Diagnostics (A 7255)
  - Alpha Amylase from Sigma Diagnostics (A 6211)
- Deionized water
- Stirring rod
- Hot plate
- Thermometer
- Autoclave or pressure cooker
- 125 mL Erlenmeyer flask
- Rubber stopper with hole and tube
- Pipette (including ones that can measure in microliters – may be substituted for a syringe that can accurately measure in microliters)
- Graduated cylinder
- Balance that can accurately weigh to the hundredth of a gram
- Centrifuge
- Parafilm
- Stir plate and stir bar
- Grease pen

Ethanol Assay Materials

- **Safety glasses**
- **Gloves**
- Ethanol assay kit from Sigma Diagnostics (catalog number 332-A)
  - NAD-ADH Single Assay Vial (individual catalog number 330-1)
  - Ethanol Standard Set (individual catalog number 332-11)
  - Glycine Buffer Reagent (individual catalog number 332-9)
- Spectrometer that can read at 340 nm
- Cuvettes or tubes appropriate for the spectrometer
- Kimwipes
- Saline
- Syringe and needle

Carbon Dioxide Analysis Materials

- **Safety glasses**
- **Gloves**
- Rubber tubing
- Ring stand and clamps
- 50 mL burette or pipette with stopper
- Bromthymol Blue *(alternative spelling Bromothymol)*
- Tube (minimum one liter)

Data Analysis Materials

- Computer with Microsoft Excel
- Printer
- Disks for data storage

PREPARATORY ACTIVITIES (ONE DAY)

Previous Knowledge and Lessons

- At this point in the semester, students should already understand the scientific method. In addition, students should know general laboratory procedures, such as taking measurements, data collecting, and record keeping. Although this laboratory will strengthen the understanding of the scientific method, it should not be a new concept, and this should not be the first time students are asked to use the scientific method for their laboratory investigations.
- Students should have an introduction to Microsoft Excel before performing the Data Analysis section of this activity. Students should be introduced to the concept of using a spreadsheet and how to convert Excel spreadsheets into graphs.
- This activity is meant to be part of a unit on metabolic pathways. It is best done in conjunction with lessons surrounding glycolysis, cellular respiration, Krebs (TCA) cycle, fermentation (anaerobic respiration), and photosynthesis. Students should understand the big concepts behind these and other metabolic pathways, especially those surrounding fermentation. This activity should enhance students' understanding of the industrial uses of metabolic pathways, with a focus on biomass conversion (fermentation of corn to ethanol).

DAY ONE - INTRODUCTION AND LABORATORY PREPARATION

Part One - Introduction

15 minutes

- Review of metabolic pathways with an emphasis on fermentation and anaerobic respiration.

30 minutes

- Class discussion: How do we use the metabolic pathways of other organisms (especially microorganisms like bacteria and yeast)? Answers may be written on an overhead, the board, or in student notebooks. The teacher should facilitate this discussion by guiding students to appropriate answers.
- Discussion should be wrapped-up with an emphasis on biomass conversion. This is a good lead-in to the laboratory investigation.
- At times, the teacher should suggest a few uses that the students may not think of or elaborate on student answers…

Food industry
- beer, wine, root beer
- vinegar
- yogurt, cottage cheese, cheese, custard, butter
- sauerkraut
- breads
- sausage, pepperoni, salami
- uses yeast, bacteria
- uses enzymes:
  - chymosin for cheese production
  - amylase to break down starch
  - glucose isomerase to get sweeter products
  - pectinase to clarify fruit juices
  - glucose oxidase to dry egg whites

Drug industry
- organism produces drug as a by-product of metabolic functions
- antibiotics (Penecillin)
- vitamins (A, B2, B12, Biotin, C)

Chemical industry
- acids (lactic acid, acetic acid)
- alcohols (ethanol)
- others (cellobiose, glucose, xylose, arabinose, xylitol, glycerol) Symbiotic relationships
- digestion
- lactose-intolerance

Biomass conversion
- plant matter
- corn to ethanol (LEAD IN TO LAB EXPERIMENT)

Part Two – Laboratory Preparation

5 minutes

- Overview of three-day laboratory investigation. Remind students that on the third day they will be meeting in the computer lab for data analysis.
- Hand out instructions for the laboratory.
- Review vocabulary as needed.

40 minutes

- Students work in pairs and follow instructions for the first fermentation set-up.
- Students work in pairs to design their own fermentation investigation using the scientific method. Before the fermentation is set-up, students report hypothesis and variables to instructor for verification.
- Students keep notes on experiment design in laboratory notebooks and answer questions in the laboratory handout.

MAIN ACTIVITIES

DAY TWO – IDENTIFICATION AND QUANTIFICATION OF FERMENTATION PRODUCTS

Introduction

25 minutes

- Remind students that on the following day they will be meeting in the computer lab for data analysis.
- Warm-up activity: Ask students to write the chemical equation for the fermentation reaction they set-up the previous day. As a class, go over the reaction and allow students to brainstorm ways they could identify the products. Ask how the products might be quantified.
- Brief review of the carbon dioxide test and the ethanol endpoint assay. If students have not used a spectrometer or a centrifuge before, a brief review on how to use the device may be needed.
- Review vocabulary as needed.

Part One – Carbon Dioxide Analysis

20 minutes

- Students confirm the presence of carbon dioxide as a product in both fermentation reactions (using Bromthymol Blue).
- Students quantify the carbon dioxide production and make quick comparisons and generalizations between both fermentation reactions (using water displacement).
- Students record data in lab notebooks and answer the questions in the lab handout.

Part Two – Ethanol Analysis

45 minutes

- Students run the ethanol standards and record data in lab notebooks.
- Students confirm the presence of ethanol and quantify it for each fermentation reaction (using ethanol assay – blood alcohol kit from Sigma Diagnosis).
- Students record data in lab notebooks and answer the questions in the lab handout.

DAY THREE – DATA ANALYSIS AND MANIPULATION

Introduction

10 minutes

- Quick review of the day's activities.
- Review vocabulary and Microsoft Excel commands as needed.

Part One – Data Analysis and Manipulation

55 minutes

- Students use Microsoft Excel to create a spreadsheet for the data.
- Students use Microsoft Excel to create graphs for data interpretation.
- Students extrapolate data according to calculations.
- Students write in lab notebook: conclusions (what did they discover about their original hypothesis); ideas for further investigations – what other hypotheses can be made and how can they be tested?

Part Two – Class Discussion (Wrap-up)

25 minutes

- Students share their own investigations as well as their findings from the data analysis. The class discusses general conclusions about the fermentation process and what variables affect the quantity of ethanol and carbon dioxide production.
- Students share ideas for further investigations.

EXTENSIONS

- Students may research the industrial uses of metabolic pathways of other organisms (see list from preparatory activities).
- Websites with experiments on food production using fermentation:

http://www.uwrf.edu/biotech/workshop/activity/act1/act1.htm
http://www.lcsc.edu/ns172/Outlines/fermenthome.html
http://www.wsu.edu:8080/~hurlbert/pages/101lab16.html
http://www.inform.umd.edu:8080/EdRes/Topic/AgrEnv/ndd/4h/

- Allow students to perform further investigations based on answers to laboratory questions.
- Talk about the uses of the ethanol assay for testing blood-alcohol levels.