**Metabolite Production During Fermentation to Produce Kombucha**

**Experimental Purpose-** To measure the rate of production of acetic acid and ethanol during Kombucha production and determine what factors affect this rate.

**Skills to be developed**

* Use of nmr to follow a reaction
* Graphing substance amounts over time to determine rate of reaction

**Learning Goals**

* Microorganisms as chemical “factories”
* Anaerobic metabolism
* Carbohydrate metabolism
* Enzyme kinetics

**Background reading**

Fermentation is a metabolic process catalyzed by enzymes provided by a microorganism. The process is usually anaerobic (in low oxygen concentrations) and stores some of the energy of the sugar molecule in ATP. Smaller substances such as methane, hydrogen, ethanol, and lactic acid (amongst others) are produced.

Fermentation occurs in the gastrointestinal tracts and in muscles of all animal (including humans). It is used in food production. Kombucha, pickled cucumbers, yogurt, and kimchi all undergo fermentation to produce lactic acid which gives these foods their sour taste. Alcoholic beverages are produced via fermentation and bread dough rising is caused by the enzymes in yeast which produce ethanol and carbon dioxide.

Glycolysis is the metabolic breakdown of glucose to form pyruvate, a three-carbon molecule. Under normal conditions pyruvate is decarboxylated releasing carbon dioxide and an acetyl group which is attached to coenzyme A and then further metabolized through the Krebs cycle. However, under anaerobic conditions pyruvate is converted to lactic acid or ethanol depending on the organism.1

Kombucha is a fermented beverage. It is produced from sweetened tea (or coffee) by adding a symbiotic culture of bacteria and yeast (SCOBY). The yeast produces ethanol and carbon dioxide gas which gives it an effervescent quality. The bacteria metabolize the ethanol to form acetic acid. Both types of microbes also produce other metabolites including vitamins, organic acids, and even proteins.

Several different experiments for undergraduates are based on following the products of fermentation. Recent examples include the use of HPLC and titration to analyze Kombucha2, observation of color changes in red cabbage fermentation to indicate pH changes3, use of ethanol and gas probes to follow yeast catalyzed sugar fermentation4, and measuring gas volume produced with a homemade water displacement gasometer5.

We will make Kombucha and follow the production of two metabolites (ethanol and acetic acid) over the course of several weeks. In one part of the experiment we will use ethanol vapor and carbon dioxide vapor probes mounted above the fermenting tea solution along with a pH probe set into the solution. Another part of the experiment involves using nmr to analyze the amount of ethanol and acetic acid produced. Integration of peaks specific to the metabolite of interest compared to the size of the water peak integration will give the amount of metabolite present.

Figure 1. pH, CO2, and ethanol produced during Kombucha fermentation.

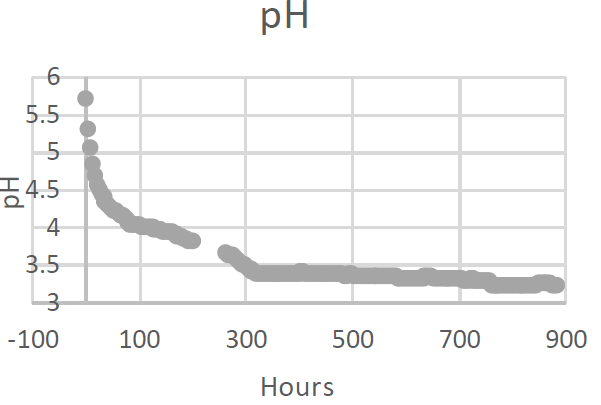
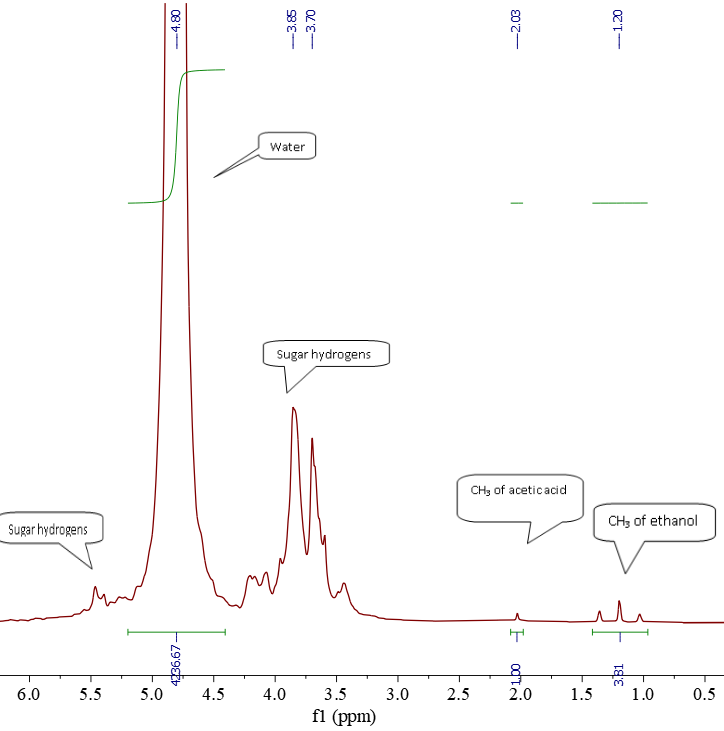
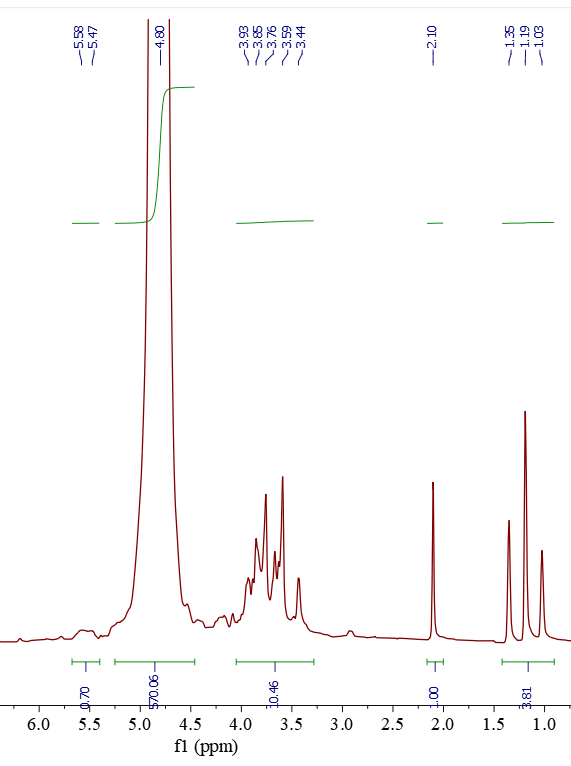


Figure 2a. nmr of Kombucha beginning to form Figure 2b. nmr of Commercial Kombucha Tea

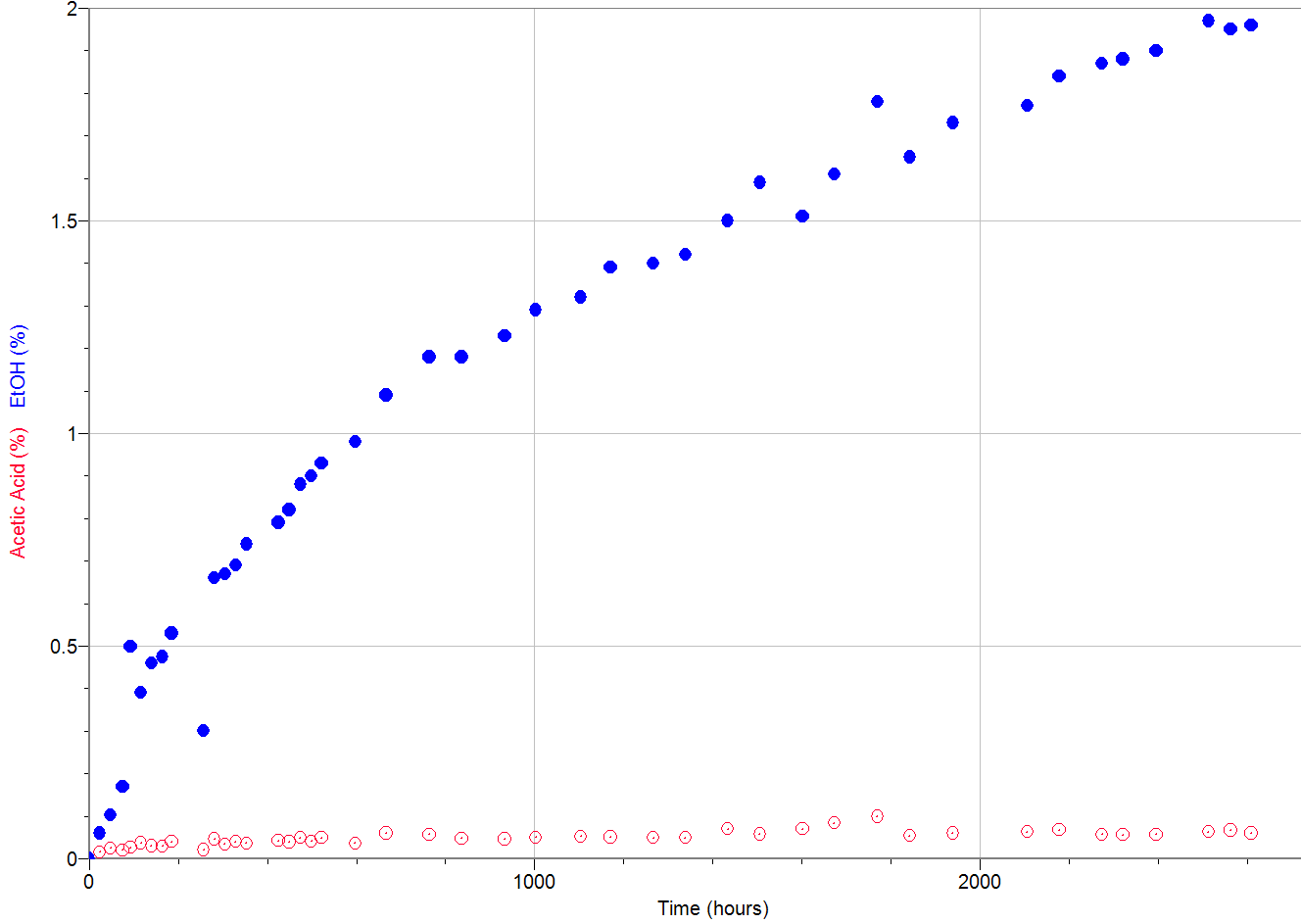
The nmr in Figure 2a has peaks for water (4.80 ppm) and sugar (5.5 and 3.8 ppm) with just a trace of acetic acid at 2 ppm and the CH3 of ethanol at 1.2 ppm (the CH2 of ethanol is under the sugar peak at 3.8 ppm and the OH is at the same place as water). Other chemical substances such as flavor molecules, caffeine, and vitamins are present is such small amounts that their peaks are not seen.

The calculations for the analysis of Commercial Kombucha tea (Figure 2b) is:

Acetic acid (CH3) at 2 ppm integrates to 1.00, so 1.00/3 gives 0.33 as the integration value of one hydrogen. Ethanol’s CH3 at 1.2 ppm integrates to 3.81; dividing by three gives 1.27 for one hydrogen of ethanol. The peak for water at 4.8 ppm integrates to 570.06. Since the OH from ethanol is also part of that and contains one hydrogen, we subtract the value of one ethanol hydrogen (1.27) to get 568.79. Dividing by two gives 284.395 as the integration value of one hydrogen of water. So, we have a ratio of acetic acid to ethanol to water as 0.33 to 1.27 to 284.395. Totaling and then dividing each by the total converts to 0.12% acetic acid and 0.45 % ethanol. These are molar ratios.

Meet with a group to design an experiment. You will need to make a batch of Kombucha and then develop some variation of this to test. The fermentations will be followed by placing the mix (about 1 mL) along with a tiny amount of the SCOBY in nmr tubes. Make one control experiment and at least one variation experiment. Obtain an nmr spectra every day for 10 days, and then once or twice per week afterwards. Perform calculations on each nmr spectra and then graph the ethanol and acetic acid produced (%) versus time (hours).

Figure 3. Graph of Decaf Coffee Kombucha



The graph could be used to predict the time needed to produce the best flavor and not let too much sour acetic acid form. It can also show when the sugar is used up or the SCOBY is no longer functioning. In addition, the results of the variation you do might help predict best conditions for Kombucha. This could include type of sweetener, use of sealed cap to trap CO2 or vented cap to release it, temperature, removal of oxygen, presence of light, or many other possibilities.

Write out the question/purpose of your variation: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**Table of Reagents** (This is for the Kombucha preparation)

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| --- | --- | --- |
| Reagent | Amount | |
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|  |  |  |
|  |  |  |

**Procedure Observations\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

*(use the standard method of recording procedure and observations as you do the experiment)*

**Post Lab-Calculations**- Show calculations (one example here) and others written on the nmr spectra.

**Results:** Attach nmr spectra (samples), data tables and graphs. Include the storage location of the data files.

**Conclusion:** Give a statement to answer the purpose. Use data (numbers from the experiment) to back up your conclusion and explain how they support your answer.

**References:**

1. J. Hill, S. Baum, and R. Scott-Ennis, Chemistry and Life; An Introduction to General, Organic, and Biological Chemistry, 6th ed., Prentice-Hall, Upper Saddle River, NJ (2000), pp. 705-708.
2. B. Miranda, N. Lawton, S. Tachibana, N. Swartz, and W. Hall, Titration and HPLC Characterization of Kombucha Fermentation: A Laboratory Experiment in Food Analysis, *J. Chem. Educ.* 2016, **93**, 1770-1775.
3. J. Linder, et. al., Exploring Acid-Base Chemistry by Making and Monitoring Red-Cabbage Sauerkraut: A Fresh Twist on the classic Cabbage-Indicator Experiment, *J. Chem. Educ.* 2019, **96**, 304-307.
4. Sugar Metabolism with Yeast (Ethanol)<https://www.vernier.com/experiment/bio-i-10b_sugar-metabolism-with-yeast-ethanol/>, accessed December 21, 2020.
5. Sugar Fermentation by Yeast, <https://www.vernier.com/experiment/chem-i-24_sugar-fermentation-by-yeast/>, accessed December 21, 2020.
6. R. Weinberg, Measuring Yeast Fermentation Kinetics with a Homemade Water Displacement Volumetric Gasometer, *J. Chem. Educ.* 2018, **95**, 828-832.