Case Study I—Soy Sauce.

Scenario:

Brewing soy sauce is one of the original biotech industries. Soy sauce was shipped in barrels within Asia over 500 years ago, and in bottles to Europe by the 1600s. Now soy sauce is used all over the world.

About 5000 years ago in China, people grew soybean crops for food and animal feed. Because soybeans spoil easily, salt was added as a preservative. Over time the beans fermented much like pickles or sauerkraut. Unlike pickles, however the soy beans turn into a paste called miso as they ferment. The paste is easier to digest than the unfermented soy beans, and people have been eating it for centuries. About 500 years ago, someone discovered that instead of discarding the sauce at the bottom of the barrels, they could use it for cooking. Thus, soy sauce was invented.

Unlike making wine from grapes, soy sauce brewing is performed in two stages. First, the soy beans are steamed and mixed with toasted crushed wheat. Fungi Aspergillus oryzae and Aspergillus sojae are added to the mixture to make koji (the first step in the soy sauce-making process) that is then left uncovered for a couple of days.

Next, salt and water are added to koji to form a mash called moromi. Moromi is then put in airtight containers where it is allowed to ferment for at least 6 months. The mash is then squeezed to get the liquid soy sauce. Finally, the sauce is filtered, pasteurized, and tightly bottled for distribution.

Questions

I. Koji phase

1. In the koji phase, the mixture is inoculated with two species of Aspergillus fungi, and the mixture is left uncovered.

   i) If you analyze the microbial populations found in koji, what do you expect to find?
      A. Aspergillus fungi only
      B. Aspergillus and other fungi
      C. bacteria only
      D. various fungi (including Aspergillus) and bacteria
      E. no microbes

   ii) If you expect to find microbes other than the ones with which the mixture was inoculated, explain where they come from and why they thrive in koji. If you do not expect to find additional populations, explain why not. Because the koji mixture is left uncovered, microbes other than what the mixture was inoculated with have a chance to fall into the vats. And because many nutrients are available in this complex mixture, these various species can thrive in koji.
2. In the koji phase of soy sauce production, the content of the bean/wheat paste and the number of fungi change. These two processes are
   A. related to each other in that the rate of one is dependent on the rate of the other
   B. related to each other in that both are caused by the same third process
   C. unrelated to each other

3. What reactions/processes (if any) are responsible for changes described in question 2 above?
   Macromolecules found in koji are broken down via hydrolysis reactions, and some of the glucose generated is utilized through glycolysis and respiration by the various organisms in the mix to produce ATP for metabolism and reproduction.

4. Where in the cell do these reactions/processes occur?
   The hydrolysis reaction that break down macromolecules actually happen outside of the cells. Enzymes secreted by the two Aspergillus species are particularly important in catalyzing the breakdown of macromolecules. Glycolysis proceeds in the cytoplasm of individual cells, citric acid cycle and ETC occur in the mitochondria.

II. Moromi phase

Once Aspergillus has broken down the macromolecules in the soybeans and wheat into monomers, the koji phase ends. Moromi is then made by mixing koji with water and enough salt to make a 16-20% concentrated salt solution, or brine.

1. When brine is added, the populations of microbes found in koji change. Do you expect greater or lesser microbial diversity?
   A. Greater
   B. Lesser

2. How does adding brine lead to these changes?
   Most microbial species can’t survive at high salt concentrations because osmosis causes water from inside the cell (low salt environment) to leak out into the high salt environment of the outside.

3. Another challenge to the microbes in moromi is that it is placed in airtight containers for a number of months. What types of microbes will survive under these conditions?
   Organisms that are either always anaerobic in their metabolism or those that can switch to anaerobic metabolism.
   Explain how they obtain energy for life processes.
   Glycolysis does not require oxygen, so organisms use glycolysis to derive their ATP. But because this type of metabolism requires that NADH molecules are recycled back to NAD⁺, pyruvate is fermented to either lactic acid or ethanol in the reaction that recycles NADH back to NAD⁺.
4. Both lactic acid and ethanol are found in soy sauce after the moromi phase is complete. At a minimum, how many species of microbes thrive in moromi?
   A. 0  
   B. 1  
   C. 2  
   D. 3  
   E. can’t tell  

   Justify your answer.  
   Fermentation pathways end up with either lactic acid or ethanol as waste products. Because both are present in moromi, we know that both types of fermentation have to be performed by organisms in the mixture, i.e. there are at least two types of microbes at work here.

5. Lactic acid and ethanol are produced from the same starting material.
   i) What is the starting molecule?
      A. Glucose  
      B. Pyruvate  
      C. ATP  
      D. NADH  
      E. CO₂  
      F. Other  

   ii) What other waste products are produced along with lactic acid and/or ethanol?
      In the ethanol fermentation, pyruvate is first converted to a substance that can be converted to ethanol, releasing CO₂ as a waste product along the way.

   iii) Do lactic acid and ethanol result from oxidation or reduction of their respective precursors?
      A. Lactic acid and ethanol both result from oxidation of the precursors  
      B. Lactic acid and ethanol both result from reduction of the precursors  
      C. Lactic acid results from oxidation, while ethanol results from reduction  
      D. Lactic acid results from reduction, while ethanol results from oxidation

III. Alcohol concentrations

Fermentation easily occurs without human intervention. For example, tiny breaks on the skin of ripened grapes allow entry of microbes. Fermentative yeasts thrive in the interior of the grape, which provides both the high concentration of sugars and low pH. Yeasts metabolize the grape sugars for energy, and the waste products—carbon dioxide and ethanol—are rapidly transported out of the cell.

In an artificial fermentation, such as making wine, the process is carried out in a manner that permits CO₂ to escape while preventing the entry of O₂. There, alcohol continues to
build up until the alcohol tolerance level of the specific yeast population is reached, ending the fermentation cycle. The figure below shows the results from a simulation of wine fermentation over a 10-day period.

1. What product of the fermentation that produces the ethanol is not shown in the graph?
   CO₂ is the waste product not shown in the graph.

2. Notice that not all grape sugar is converted to ethanol. Why not?
   At some point the concentration of ethanol produced by the yeast becomes so high that the yeast can no longer tolerate it and the yeast population dies out. If as is the case in this graph, unutilized sugar remains when the yeast cross their ethanol tolerance threshold and start dying, the unutilized sugar will remain in the mixture. If, as shown above, the fermentation stops before all the sugar is used up, a sweet wine will be produced. If the fermentation continues until virtually all the sugar has been used up, a “dry” wine will be produced.

3. If you removed the alcohol as it was produced, would you predict an increase or a decrease in the amount of grape sugars at ten days? An increase or a decrease in the population of yeast at ten days?
   A. No change in either sugar or yeast
   B. Increase in sugar, no change in yeast
   C. Decrease in sugar, no change in yeast
   D. Increase in sugar, decrease in yeast
   E. Decrease in sugar, decrease in yeast
   F. Increase in sugar, increase in yeast
   G. Decrease in sugar, increase in yeast
4. A bottle of wine may spoil if it is allowed to sit for some time after being opened or if its cork does not form a tight seal.

   i). Does the amount of grape sugar left in the wine change if the wine spoils?
      A. No, the amount of sugar does not change if the wine spoils.
      B. The amount of sugar increases if the wine spoils.
      C. The amount of sugar decreases if the wine spoils.

   ii). Explain what causes the wine to spoil under these conditions.
    *Contamination from airborne microbes results in new metabolic activity.*
    *Remaining grape sugar is broken down by these newly arrived organisms either through lactic acid fermentation or through citric acid cycle and oxidative phosphorylation (ETC).*