

## ABSTRACT

OLSEN, MAEGAN JESSIE. Redox Potential Trends of Cucumber Fermentation as Influenced by Microbial Growth and Gas Purging. (Under the direction of Dr. Ilenys M. Pérez-Díaz.)

Determining the redox potential of a system can be a useful tool in evaluating the thermodynamic changes that occur over a period of time. Currently, fermentation industries use pH measurements to assess the progress of fermentation and growth of lactic acid bacteria (LAB). Redox potential ( $E_h$ ) measures the transfer of electrons within a system, rather than solely the concentration of hydrogen ions, as measured by pH. Continuous monitoring of fermentation by  $E_h$  may allow for more descriptive analysis of the metabolic process and could offer a method for the earlier prediction of spoilage by yeasts. The trends in redox potential of fermenting cucumbers were observed to evaluate the possible application of this parameter in monitoring the development of fermentation. Additionally, an evaluation of the effect of gas purging on microbial growth during the fermentation was conducted using redox potential trends as a monitoring tool. Cucumbers were packed and brined using sodium chloride, calcium chloride, and acetic acid and the redox potential monitored during fermentation. Examination of both pasteurized-inoculated jars and non-pasteurized jars was conducted in this study. In addition, changes in redox potential were measured in fermentations that were purged with nitrogen, oxygen, hydrogen, or left untreated. Brine samples were analyzed for microbial counts, pH and substrates and products of fermentation. Under conditions representative of a standard cucumber fermentation, a dramatic decrease in the redox potential was observed during the first day,

concomitantly with the initiation of the log phase of bacterial growth. However, in the presence of spoilage yeast, redox potential remained low over this initial period. The progress of a fermentation sparged with nitrogen, oxygen, or no treatment produced similar  $E_h$  trends. The  $E_h$  trend was more reduced under hydrogen sparging. A heightened growth rate for LAB, more efficient production of lactic acid, and lower production of ethanol occurred with nitrogen sparging. The evidence suggests that the growth of yeasts in a cucumber fermentation could be detected based upon differences in the redox potential. Distinct variations in  $E_h$  were still recorded after pH values decreased and remained constant, suggesting the valuable possible application of  $E_h$  to monitor industrial cucumber fermentations. Additionally, changes in redox potential were affected by gases introduced during cucumber fermentations and sparging with nitrogen could have substantial benefits to the industry.

Redox Potential Trends of Cucumber Fermentation as Influenced by  
Microbial Growth and Gas Purging

by  
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**Chapter 1.**  
**Literature Review**

## **1.1. Cucumber Fermentation**

For thousands of years, foods and beverages have been fermented as a means of preservation. Accidental at first, the art of fermentation was cultivated over time to become the predictable, established process it is today. Not until 1857 did the French scientist Louis Pasteur conclude that microorganisms were essential for fermentation to proceed (Dubos 1998). This technology is vital in many cultures worldwide and is used in the production of foods such as cheese, meat, and bread (Hutkins 2006). Fermentation processes not only extend shelf-life in vegetable products as kimchi and sauerkraut, but impart desirable flavor attributes to the final product (Breidt 2006; Breidt and others 2007). The nutritional content of a food may also be enhanced as a result of fermentation. Complex, indigestible polymers can often be catabolized through fermentation to digestible, useful carbohydrates. Liberation and synthesis of previously unavailable nutrients can also be a benefit of the fermentation process (Potter and Hotchkiss 1998).

Specific fermentations are performed depending upon the microorganism involved. Yeasts, which are eukaryotic organisms, primarily carry out ethanol fermentations, converting glucose to ethanol. Lactic acid bacteria (LAB), which are prokaryotes, primarily ferment glucose to lactic acid. Both yeasts and homofermentative LAB utilize the Embden-Meyerhoff-Parnas pathway, while other LAB are heterofermentative and use the phosphoketolase pathway to produce lactic acid, carbon dioxide, ethanol, and acetate (White 2007).

Lactic acid bacteria are intrinsically present in vegetables, plant material, the gastrointestinal tracts of humans and animals, and dairy products among other foods and have gained GRAS (Generally Recognized As Safe) status. Consequently, LAB may be added to minimally processed vegetable products intended for consumption. Cucumber fermentation is a technology that significantly benefits from the assigned GRAS status (Caplice and Fitzgerald 1999). The natural succession of microbial populations in cucumber fermentations begins with high levels of aerobic bacteria, sometimes including pathogenic and spoilage organisms (Breidt 2006). Relatively low numbers of LAB are naturally present initially, but are able to outcompete the other organisms early during the fermentation due to their ability to survive in extreme environments characterized by high acid and salt concentrations (Breidt 2006; Hutkins 2006).

The pickle industry is dominated by the production and sale of acidified, or fresh-pack, pickles (Fuller and Dull 1983), rather than traditional fermented pickles. Roughly 70% of pickles produced commercially are acidified or refrigerated (Breidt 2006). Cucumbers are packed fresh without fermentation or heat processing, covered with brine containing preservatives and salts and pasteurized when manufacturing acidified pickles (Breidt 2006; Fuller and Dull 1983). The pasteurization step ensures safety by eliminating harmful microorganisms that may be present in the product, which could include enteric pathogens (Breidt 2006). Pasteurization also prevents spoilage by naturally occurring LAB or other microflora, and inactivates enzymes in the product that could lead to undesirable softening of the plant material (Breidt 2006). The pH value for an acidified pickle is around 3.7, containing 1 to 2% acetic acid and 2 to 4% sodium chloride (Breidt and others 2007).

Similar to fresh-pack pickles, refrigerated pickles are another popular commodity. These are acidified as described previously, but are not heat pasteurized, and require refrigeration (Hutkins 2006). Both fresh-pack and refrigerated pickles offer a crisp, fresh tasting product.

Fermented pickles are most popular to consumers as hamburger dill chips. To begin the production of fermented pickles, washed and sorted cucumbers are stored in open bulk tanks, 8,000 to 10,000 gallons in size. The pickle tanks are often outside and are left uncovered, allowing UV radiation from the sun to eliminate yeasts present on the surface of the brine (Breidt and others 2007). Equilibrated brine cover solution containing around 5 to 6% sodium chloride, 0.1 to 0.4% calcium chloride, and 0.05 to 0.2% acetic acid provides desirable conditions for LAB, such as *Lactobacillus plantarum*, to predominate during cucumber fermentation (Breidt 2006; Breidt and others 2007). Since cucumbers are mostly composed of water (Hutkins 2006), the added salt in the brine cover solution draws water and sugars from the cucumber fruit, which further aids to initiate the fermentation (Jay and others 2005). *L. plantarum* utilizes the available carbohydrates, such as glucose and fructose, reducing the pH by lactic acid production. Fermented cucumbers can be stored in the tanks for a year or longer after the fermentation process. Firmness is maintained throughout the lengthy storage process by the use of salt and calcium chloride in the brine (Buescher and others 1979). After fermentation and storage, the cucumbers are removed from the tanks, desalted in water, packed, and covered in fresh brine cover solution and seasonings. Similar to acidified pickles, the final pH for fermented pickles stays around 3.2 to 3.7 (Breidt and others 2007; Hutkins 2006).

During cucumber fermentation, the production of carbon dioxide can sometimes be a result of the presence of coliforms, yeasts, or heterofermentative LAB. Excessive CO<sub>2</sub> production can lead to bloater pickles, which have undesirable gas pockets in the cucumber flesh. To remove CO<sub>2</sub>, manufacturers will often purge air into the tanks (Fleming and others 1975; Hutkins 2006).

Acidified pickles are defined by the U.S. Code of Federal Regulations in 21 CFR part 114 as foods that are originally low-acid to which acid or acid food ingredients have been added. The product is characterized by having a water activity above 0.85 and a final pH of 4.6 or below. Acid foods are defined as those that have natural pH values of 4.6 or below (Breidt 2006). The pathogen of concern in anaerobic, acid or acidified foods is *Clostridium botulinum*, which will not sporulate or produce its neurotoxin when the pH is at or below 4.6 (Ito and others 1976).

The acidic pH targeted at the end of cucumber fermentation, as well as carbohydrate exhaustion, and the high acid and high salt conditions create an environment unsuitable for the survival of nearly all vegetative bacteria. Incidence of foodborne illness due to fermented or acidified vegetables is extremely rare due to the nature of these products (Breidt 2006).

In the industry today, the majority of cucumbers are fermented naturally, based upon their indigenous microflora. In an effort to maintain reliability and control of the process, the use of starter cultures is sometimes implemented in various fermentations. Although using starter cultures is more expensive, they can offer more reproducible results and predictable fermentations (Hutkins 2006).

## 1.2. Cucumber Spoilage and Other Problems

The quality and timeline of cucumber fermentations can be monitored through pH measurements. A decrease in the value of brine pH is seen along the course of cucumber fermentations, as organic acids, chiefly lactic acid, are produced by LAB. Characteristic flavors develop during this process, as sourness increases and sweetness decreases (McFeeters 2004).

Following efforts to reduce the amount of salt used for cucumber fermentations, a decrease in fruit firmness is often observed (Breidt and others 2007). The inclusion of calcium prevents cucumber softening (Fleming and others 1978). Pickle softening occurs repeatedly as a consequence of spoilage bacteria and fungi being present. These organisms produce pectinesterase and polygalacturonase, which hydrolyze pectin in the cucumber cell wall during fermentation (Bell and Etchells 1956; Bell and others 1950; Breidt and others 2007).

Spoilage by propionic and butyric acids has been documented in the fermentation of cucumbers. Fleming and others reported spoilage after an anaerobic lactic acid fermentation was completed using a decreased salt concentration (2.3%) (Fleming and others 1989).

Propionic acid, butyric acid, acetic acid, and n-propanol were produced during storage.

Lactic acid levels generated during fermentation decreased from 140.1 mM to non detectable post-spoilage.

Occasional red-colored cucumber spoilage has been reported in the pickling industry.

*Lactobacillus casei* and *Lactobacillus paracasei* in the presence of FD&C yellow no. 5, also

known as the azo dye tartrazine, were determined as the causative agents (Pérez-Díaz and others 2007). Deposits of red-color appeared on cucumber flesh as *L. casei* and *L. paracasei* grew and utilized the tartrazine present as a brine coloring agent. *L. casei* and *L. paracasei* are not the dominant LAB in natural cucumber fermentations, instead, mainly *L. plantarum* is found. *L. casei* has been linked to causing spoilage in various fermented foods and beverages (Khalid and Marth 1990). The addition of the preservative sodium benzoate or the use of turmeric in place of tartrazine may prevent the development of the red-colored spoilage.

The occurrence of bloater cucumbers during pickle fermentation has been a re-occurring problem for the industry. Although gas-producing heterofermentative LAB, coliforms, and yeasts may be involved in the bloater formation (Etchells and others 1968); the primary causative agent is homofermentative lactobacilli capable of converting malic acid to CO<sub>2</sub> and lactic acid (McFeeters and others 1982). Cucumber bloating is greatly reduced if nonmalolactic homofermentative lactobacilli are used as starter cultures; however, complete fermentation does not proceed, as evidenced by the significantly high carbohydrate concentration that remains (McFeeters and others 1984).

### **1.3. pH**

pH is a measurement of the concentration of hydrogen ions in a solution. The pH scale extends from pH 0.0, most acidic, to pH 14.0, most alkaline. Since the pH scale is logarithmic, a 10-fold change in hydrogen ion concentration is indicated by a one unit change in pH (Madigan and others 2003). The majority of microorganisms prefer a neutral pH range

from about 6.6 to 7.5, while most LAB grow at a pH of 4.0 to 4.5 (Jay and others 2005).

Bacterial cells often have mechanisms to maintain a neutral internal pH ( $\text{pH}_i$ ) regardless of the external pH. Susceptible microorganisms are not able to survive with a low  $\text{pH}_i$ , resulting in cell death. Certain organisms, however, have the enzyme ATPase which will pump hydrogen ions out of the cell, ensuring the  $\text{pH}_i$  remains neutral so they can reproduce and survive in low pH environments (Lund and others 2000).

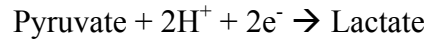
The proton motive force (PMF) in a cell is the force generated by a gradient of protons and a membrane potential, which results in potential energy that can be used in the synthesis of ATP (Prescott and others 2005; Stanier and others 1986). During respiration, protons are moved out of a cell through electron transport, and PMF is created. In fermentative organisms, such as LAB, ATP synthesis is responsible for driving the PMF (White 2007). ATPase in LAB maintains homeostasis by pumping protons out of the cell (Jay and others 2005).

## **1.4. Redox Potential**

### **1.4.1. Background**

Oxidation-reduction (redox) reactions involve the loss of electrons (oxidation) by one substance and the gain of electrons (reduction) by another. The substance being oxidized in a conjugate redox pair is referred to as the electron donor, whereas the substance being reduced is the electron acceptor (Madigan and others 2003). The electron donor is referred to as the reductant and the electron acceptor as the oxidant (Srinivas and others 1988). Pyruvate acts

as the electron acceptor and lactate as the electron donor in the reduction of pyruvate to lactate (Prescott and others 2005).



The flow of electrons in a redox system generates the energy microbial cells need to do work and survive. As electrons move from metabolic intermediates to electron carriers, they are donated to acceptors that have stronger electron affinities. Energy for the cells is released through the electromotive force that is created (Lehninger and others 1993).

The tendency for a chemical substance to be oxidized or reduced is its redox potential ( $E_h$ ).

The standard reduction potential ( $E_0$ ) is the affinity of the electron acceptor in a pair of electrons in a conjugate redox pair under standard conditions (Lehninger and others 1993).

Similarly, the redox potential of more complex environments, such as a microbial culture, are a reflection of the capacity of the system to donate or accept electrons and, therefore, of the overall energy available for work. The redox potential of a microbial culture at a given time is an indication of the metabolic and physiological state (Reichart and others 2007).

Extracellular redox potential is measured in volts (V); therefore the electric potential for microorganisms is expressed in millivolts (mV). On a scale from +800 mV to -800 mV, the +800 mV reading represents the most oxidized substance with the strongest tendency for accepting electrons. Similarly, the -800 mV reading represents the most reduced substance with the greatest tendency to donate electrons (Madigan and others 2003).

Redox potential measurements can be manipulated and are dependent upon the environmental conditions and microbial surroundings. Various gases and chemicals can be used as reducing and oxidizing agents. Reducing agents can include hydrogen gas or active metals, which donate electrons. Oxygen gas or sulfuric acid can be used as oxidizing agents. Gases are often used over chemicals or powdered agents because these do not alter the medium composition and thus do not alter the ability of microorganisms to proliferate. Oxygen gas is a strong oxidizing agent, which can accept electrons and hydrogen ions readily and show a high, positive redox potential (Jay and others 2005). For example, addition of oxygen gas into a solution will cause an increase in the redox potential (Delfini and Formica 2001). Ouvry and co-workers (2002) successfully manipulated redox potential in a culture of *Lactobacillus plantarum* in modified deMan, Rogosa, and Sharpe (MRSm) medium using nitrogen and hydrogen gases. Redox values in the medium reached  $+350 \pm 30$  mV after sparging with  $N_2$  and  $-300 \pm 30$  mV with  $H_2$ . *L. plantarum* grew and lowered the Eh of the medium from +420 mV to -40 mV. This demonstrated the consumption of oxygen and production of reducing molecules, such as sulfhydryl compounds, by the LAB (Ouvry and others 2001; Ouvry and others 2002; van Dijk and others 2000). Brasca and others (2007) also noted the strong reducing power of *L. plantarum* when inoculated in milk.

Redox electrodes are normally used to measure the redox potential of a system. Dyes can also be used; however, they offer less exact measurements and can be toxic or inhibitory towards cells and enzymes in the system (Berovic 1999; Kjaergaard 1977). Redox probes are often made of glass and have a tip made from a noble metal such as gold or platinum, which works to bind active electrons in the solution and send a signal to the probe. The

signal is amplified from the probe through a voltmeter, which gives a numerical millivolt reading (Kjaergaard 1977). Most redox probes have a silver/silver chloride reference electrode and use a 3M potassium chloride solution as reference electrolyte. Saturated solutions of quinhydrone plus pH 7.0 and pH 4.0 buffers are used for calibration of the redox probes (Berovic 1999; Kjaergaard 1977). Quinhydrone works by trapping electrons in the buffers and gives more stable standard millivolt solutions (Banhidi 2007).

The redox potential of an environment changes in the presence of microorganisms, similar to pH. Aerobes have the ability to lower the  $E_h$  of a system by utilizing the oxygen present. This functions by decreasing the amount of oxidizing agents, consequently increasing the amount of reductants available. In anaerobic environments, a buildup of reducing agents such as hydrogen gas will also cause a decrease in  $E_h$  (Jay and others 2005). Hydrogen gas is a metabolic byproduct of some microorganisms, including some involved in fermentation.

Measured and standard redox potentials are directly related through the Nernst equation (Lehninger and others 1993). The equation states:

$$E = E_o + \frac{RT}{nF} \ln \frac{[ox]}{[red]}$$

Where;

$E$  = reduction potential

$E_o$  = standard reduction potential

$R$  =  $8.315 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$  (gas constant)

$T$  = absolute temperature (K)

$n$  = number of electrons transferred in the reaction

$F = 96,485 \text{ C/mol}$  (Faraday constant)

ox = oxidant

red = reductant

Both fermentation and respiration result in the production of ATP to be used by microorganisms. Respiration is much more energetically efficient than fermentation and is the preferred method of pyruvate breakdown in organisms with both capabilities. During respiration, 38 ATP molecules may be produced from 1 glucose molecule due to oxidative phosphorylation, whereas only 2 ATP molecules are produced during fermentation. Redox reactions during fermentation differ from aerobic respiration because they occur without oxygen as the terminal electron acceptor. In lactic acid fermentation, glycolysis produces pyruvic acid, which acts as an electron acceptor and is reduced to form lactic acid (Hutkins 2006).

#### **1.4.2. The Use of Redox Potential in Industry**

Information regarding the condition of a microbial culture may be obtained using observed redox potential values (Kjaergaard 1977). When investigating redox potential during sauerkraut fermentation, van Dijk and researchers in the Netherlands (2000) reported variable trends representing different stages of fermentation and changes in sugar and organic acid content. There are two distinct fermentation phases that occur in sauerkraut fermentation. It begins with a heterofermentation initiated by salt tolerant *Leuconostoc mesenteroides* subsp. *mesenteroides*, which is the predominant LAB initially, and is succeeded by the proliferation of homofermentative *Lactobacillus plantarum*, which is more apt to tolerate highly acidic

conditions (Lu and others 2003). This group found that the initial redox potential of cabbage juice was close to +200 mV. This value fell dramatically with fermentation, reaching as low as -273 mV, after 5 to 7 days of microbial growth, and reached a plateau after roughly 10 days (van Dijk and others 2000). Fulde and Fabian (1955) also discovered significant differences in redox potential depending on the microorganisms present during sauerkraut fermentation. The strong reducing power of *Enterobacter cloacae* (referred to as *Aerobacter cloacae*) was evident 5 hours after the initiation of fermentation and reached a value as low as -200 mV within 10 hours. The presence of *E. cloacae* could be differentiated from the presence of LAB, including *L. plantarum* and *Leu. mesenteroides*.

Bourel and others (2003) observed that an environmental redox potential of -400 mV affected the growth and physiology of *Leu. mesenteroides*. The low  $E_h$  of the medium was established using hydrogen gas. Decreasing the  $E_h$  from +200 mV to -400 mV resulted in a 5 hour increase in the duration of the lag phase of growth, giving cells time to acclimate to their new conditions. The growth rate and efficiency of *Leu. mesenteroides* were also shortened under a hydrogen versus nitrogen atmosphere. However, Kaneko and others (1987) saw that the production of acid was increased under reducing conditions for a liquid concentrated culture of *Lactobacillus delbrueckii* subsp. *bulgaricus*. 0.1% sodium ascorbate was added to the culture and reacted with  $H_2O_2$  naturally produced by *L. delbrueckii* subsp. *bulgaricus* to lower the overall  $E_h$ . The value was maintained by the periodic addition of  $N_2$ . Dave and Shah (1998) showed the effects of yogurt containing the reducing agent cysteine. It took an additional 6 or 8 hours for the yogurt plus 250 or 500 mg/l cysteine to reach a pH

of 4.5. There was a decrease in the rate of acid production by the probiotic bacteria *Lactobacillus acidophilus*, *Streptococcus thermophilus*, and bifidobacteria. Negative  $E_h$  measurements were representative of the presence of cysteine and a maximum low of -217 mV was observed.

While pH measurements will take into account the hydrogen ion activity, redox measurements look at the activity of electrons in a system (Kjaergaard 1977). A low pH reading during fermentations may or may not be indicative of the presence of spoilage yeasts, as they can survive in low pH environments. Alternatively, redox readings may be more discriminative than pH when various bacteria or spoilage fungi are present and therefore could have a future as another descriptive regulatory tool.

## **1.5. Microorganisms**

### **1.5.1. Lactic Acid Bacteria and *Lactobacillus plantarum* MOP3**

Lactic acid bacteria (LAB) are defined as gram-positive, non-spore forming rods and cocci that produce lactic acid (Hutkins 2006). Gram-positive bacteria stain purple when performing a gram-stain, while gram-negative bacteria stain red. Gram-positive bacteria have a strong, thick layer of peptidoglycan in the cell wall, outside of the plasma membrane, where purple crystal violet dye adheres. The gram-negative cell wall consists of an outer membrane, thin layer of peptidoglycan, and a plasma membrane (Prescott and others 2005). LAB include members from the *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Enterococcus* groups (Prescott and others 2005). These bacteria are generally

facultatively anaerobic, meaning they prefer to live without the presence of oxygen, but are able to survive when oxygen is present.

LAB are common fermentation microorganisms because of their mechanisms for survival in acidic, high salt environments and the end-products they produce through sugar metabolism. Fermentation can be defined as the catabolism of carbohydrates to obtain energy (Hutkins 2006; Potter and Hotchkiss 1998). Microorganisms require energy, which mainly comes in the form of ATP, to exist in their environment. Organic acids, mainly lactic acid and acetic acid, are generated by LAB during fermentation. The increase in acid decreases the pH in the environment and works to create surroundings that are inhibitory against both pathogenic and spoilage microorganisms (DeVuyst and Vandamme 1994). In addition to producing organic acids during fermentation, LAB can generate other inhibitory compounds such as bacteriocins and hydrogen peroxide (Breidt 2006; DeVuyst and Vandamme 1994).

The *Lactobacillus* genus is extremely diverse, with over eighty species included (Hutkins 2006). In addition to being naturally found on vegetable products, lactobacilli are seen as part of dairy foods such as yogurt and cheese, fermented beverages such as beer and wine, and breads such as sourdough. Lactobacilli are also one of the major groups of probiotic organisms, believed to have advantageous effects on gastrointestinal health. Besides being able to persist in the human GI tract, these bacteria can grow at various temperatures and in the presence of an assortment of hurdles such as high salt and high acid (Hutkins 2006).

LAB are classified as either homofermentative or heterofermentative based upon how they metabolize available carbohydrates. Lactobacilli can be grouped as obligate

homofermentors, obligate heterofermentors, or as facultative heterofermentors, meaning they have the genetic capability to use either metabolic pathway (Hutkins 2006). *Lactococcus lactis* and *Streptococcus thermophilus* are two well known obligate homofermentative LAB. Hexoses, or six carbon sugars, are broken down by homofermentors via the Embden-Meyerhoff-Parnas, or glycolytic, pathway. Aldolase catalyzes the breakdown of fructose 1,6-bisphosphate (six carbons), into two three carbon sugars, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DeVuyst and Vandamme 1994; Hutkins 2006). The trioses are then later converted into pyruvate through a series of reactions, and then reduced from pyruvic acid to lactic acid by lactate dehydrogenase (DeVuyst and Vandamme 1994; Hutkins 2006). Fermentation via the homofermentative pathway produces 90% lactic acid and two moles of pyruvate and two moles of ATP for every mole of carbohydrate utilized (DeVuyst and Vandamme 1994; Hutkins 2006).  $\text{NAD}^+$  is converted to NADH by glyceraldehyde 3-phosphate, and is later oxidized back to  $\text{NAD}^+$  by lactate dehydrogenase to preserve the NADH/ $\text{NAD}^+$  balance (Hutkins 2006).

Heterofermentative LAB can breakdown both hexose, six carbon, and pentose, five carbon, sugars via the phosphoketolase, or pentose phosphate, pathway. Obligate heterofermentative LAB include *Leu. mesenteroides* and *Oenococcus oeni* (Hutkins 2006). Hexoses can be broken down to xyulose 5-phosphate, which is then split by phosphoketolase into the three carbon sugar glyceraldehyde 3-phosphate and the two carbon sugar acetyl phosphate (DeVuyst and Vandamme 1994; Hutkins 2006). Carbon dioxide is also released during the decarboxylation of 6-phosphogluconate to ribulose 5-phosphate by 6-phosphogluconate dehydrogenase. As seen in the homofermentative pathway, lactic acid is produced from the

metabolism of glyceraldehyde 3-phosphate. Acetyl phosphate is further broken down to form acetate from acetyl CoA, or further metabolized to acetaldehyde and, finally, ethanol (DeVuyst and Vandamme 1994; Hutkins 2006). Lactic acid represents ~50% of the end-products of heterofermentative fermentation, with the remaining products comprised of carbon dioxide, ethanol, and acetate (DeVuyst and Vandamme 1994). One mole of ATP is formed from every mole of carbohydrate consumed (Hutkins 2006).

While some microorganisms can get the energy they require through respiration, LAB are classified as obligate fermentors, which do not possess electron transport proteins. To survive, LAB need to re-oxidize NADH back to NAD<sup>+</sup> (Prescott and others 2005). Rather than oxidative phosphorylation, energy in the form of ATP is created by substrate level phosphorylation reactions during fermentation (Hutkins 2006; Prescott and others 2005). In this process, organic compounds are catabolized, and high energy phosphate bonds are broken, releasing ADP which is converted to ATP. Unlike respiration, ATP is generated without the use of the proton motive force (Madigan and others 2003). However, research on *Lc. lactis*, predominantly known for its role as a milk fermentation bacterium, shows it has a working respiratory pathway (Duwat and others 2001; Gaudu and others 2002). It was under respiratory metabolism, not fermentation, that growth and survival of *L. lactis* showed more advantageous results. This contradictory story could be true for other LAB as well, but further research is needed to explore the metabolism of other important fermentative organisms (Hutkins 2006).

*L. plantarum* is a facultatively heterofermentative microorganism associated with having a dominant role in cucumber fermentations due to its tolerance for high acidity (Fleming and McFeeters 1981; Jay and others 2005). During the fermentation of cucumbers, *L. plantarum* utilizes the homofermentative pathway, producing greater than 90% lactic acid as the primary end product. LAB are also characterized by having a low G + C content, which is the percent of the DNA base pairs guanine and cytosine present. DNA with lower G + C content is characterized by lower melting temperatures since there are three hydrogen bonds that connect GC base pairs. AT base pairs are connected by only two hydrogen bonds (Prescott and others 2005). *L. plantarum* has 45% G + C content (Hutkins 2006). *L. plantarum* MOP3 is a specific strain isolated from commercial pickle fermentation.

### **1.5.2. Spoilage Yeasts and *Zygosaccharomyces globiformis***

Fungi represent a vast kingdom of eukaryotes, including both yeasts and molds. Compared to prokaryotic bacteria, fungi are very complex. Yeasts themselves are unicellular and nonfilamentous, and larger in size compared to bacteria (Hutkins 2006; Prescott and others 2005). The majority of yeasts are aerobic, however some yeasts, such as those involved in fermentation, are classified as facultatively anaerobic (Prescott and others 2005). Yeasts are able to grow in a broad range of pH's, including highly acidic pH levels (Jay and others 2005). *Zygosaccharomyces* species are known to be active fermentors of sugars (Jay and others 2005).

Yeasts also undergo an alcoholic (ethanol) fermentation to metabolize sugars via the Embden-Meyerhoff-Parnas pathway. Pyruvic acid is first converted to acetaldehyde by

pyruvate decarboxylase, and then reduced to ethanol by alcohol dehydrogenase. Carbon dioxide is formed in the process and NADH, made during the oxidation of glyceraldehyde-3-phosphate, is converted to NAD<sup>+</sup> (Delfini and Formica 2001; Madigan and others 2003).

The dominant species of yeasts associated with causing a gaseous cucumber fermentation come from the genera *Brettanomyces*, *Torulopsis*, *Hansenula*, *Torulospora*, *Saccharomyces* and *Zygosaccharomyces* (Etchells and Bell 1950; Etchells and others 1952). Bell and Etchells (1956) reported that several species from each genera of the previously identified gas producing spoilage yeasts, excluding *Saccharomyces*, did not demonstrate the ability to hydrolyze the glycosidic bonds in pectin and were not responsible for cucumber softening during fermentation. *Zygosaccharomyces globiformis* is a spoilage yeast isolated from spoiled sweet pickles by Bell and Etchells in 1952.

### **1.5.3. *Enterobacter aerogenes***

The enteric bacteria in the genus *Enterobacter* are gram-negative, facultative anaerobic, non-spore forming rods. They have a higher optimal pH, close to neutral, and higher percent G + C as compared to LAB, around 52-60%. *Enterobacter* species are able to ferment sugars by the butanediol fermentation. Glucose is metabolized to pyruvic acid, with the majority of the end products as acetoin, 2,3-butanediol, ethanol, carbon dioxide and hydrogen gas (Johansen and others 1975). Lactic, acetic, and succinic acids are also produced in smaller quantities (Madigan and others 2003).

The opportunistic pathogen *Enterobacter aerogenes* is commonly found in water, sewage and in the gastrointestinal tract of animals. It has also been linked as a causative agent of urinary tract infections (Madigan and others 2003). In 1943, Etchells and Jones isolated *E. aerogenes* as an undesirable participant in early cucumber fermentation. Commonly, it is part of the natural microflora of pickling cucumbers, but *E. aerogenes* can not sustain itself as fermentation takes place, LAB grow, and the surrounding pH decreases. Microbial counts quickly decrease within a few days of the fermentation.

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**Chapter 2.**  
**Influence of Microbial Growth on the Redox Potential Trends in  
Pasteurized Brined Cucumber Jars**

## 2.1. Abstract

Commonly, pH measurements are used during the production of fermented cucumbers to indirectly monitor acid production and the growth of lactic acid bacteria (LAB). Redox potential ( $E_h$ ) measurements, which are determined by the flux of electrons in a system, could serve as a more accurate tool to monitor the progression of the fermentation, allowing the detection of the metabolic activity and/or growth of LAB and other microbes, such as yeasts. Pasteurized and inoculated cucumber jars were evaluated for  $E_h$  changes to better understand how redox potential changes during the cucumber fermentation, and potentially how this might be used as a real time monitoring tool. Jars of diced, brined cucumbers were pasteurized and inoculated with microbes previously isolated from fermented cucumber tanks such as *Lactobacillus plantarum*, *Zygosaccharomyces globiformis*, and *Enterobacter aerogenes*, or a dual combination of them. Although an initial decrease in  $E_h$  was recorded for all jars, distinctive trends in  $E_h$  depended on inocula types. Over the 2 week fermentation, the  $E_h$  on jars inoculated with *L. plantarum*, SPYs and *E. aerogenes* increased to  $+738 \pm 123$  mV, was maintained at  $-452 \pm 25$  mV and decreased to  $-1812 \pm 429$  mV, respectively. Jars inoculated with *L. plantarum* and *Z. globiformis* had a terminal  $E_h$  value of  $-14 \pm 104$  mV, falling in between the individual measurements for jars containing each organism individually. *L. plantarum* dominated the  $E_h$  trend when inoculated along with *E. aerogenes* generating a final  $E_h$  of  $+486 \pm 72$  mV. The results indicate that changes in  $E_h$  continue to be detectable two days after pH measurements became stable. Thus,  $E_h$  may serve as a more accurate real time tool, over pH, for monitoring microbial growth and detecting undesirable yeast growth in cucumber fermentations.

## 2.2. Introduction

The practice of fermentation has been used throughout the world for centuries as a means of preservation and or improvement of food safety and quality. Fermented foods gain unique sensory qualities and an increased shelf-life. Pickles are a popular vegetable product produced through the acidification or fermentation of cucumbers. During the fermentation process, lactic acid bacteria (LAB) naturally present on the fruit will grow and produce lactic acid from sugars as the major fermentation product. Acid levels increase in the surrounding environment, causing a decline in the pH levels. The resultant removal of sugar, increased acid levels and low pH all contribute to preservation of the product. Over the course of fermentations, pH is often used to indirectly monitor growth of LAB and completion of the fermentation.

The standard commercial cucumber fermentation is characterized by the predominant growth of lactic acid bacteria and concomitant production of lactic acid. However, growth of yeasts such as *Torulopsis* spp., *Zygosaccharomyces* spp., *Brettanomyces* spp., *Hansenula* spp., *Torulasporea* spp., and *Kloeckera* spp. (Etchells and Bell 1950) may occur post-brining (Etchells 1941), producing carbon dioxide that could cause cucumber bloating (Jones and others 1941). Additionally, *Enterobacter aerogenes* has been identified as an occasional and undesired participant of the early cucumber fermentation due to its ability to produce hydrogen, which may contribute to the formation of bloated cucumbers (Etchells and Jones 1943). Although, *E. aerogenes* may reach the cucumber fermentation tanks as a member of

the natural microflora of these fruits, it can not sustain itself as fermentation proceeds and pH decreases.

Considering the potential chemical changes that may occur during cucumber fermentations, which are largely dictated by the natural microflora, and the consequences of these changes on the success of the manufacture of quality products, it is useful to develop tools that may aid in the monitoring of microbial growth through out the fermentation process. In addition to pH, redox potential ( $E_h$ ) may be used as a tool to monitor fermentations and predict spoilage. Redox potential is a measurement of a substance's ability to be oxidized or reduced. During oxidation electrons are transferred from an electron donor to an acceptor, which is reduced. While pH is a measurement of the concentration of free hydrogen ions in a system, redox potential is a measurement of the activity of electrons in a system (Kjaergaard 1977). This may provide a different, broader picture of the electron transfer during fermentation processes and may detect growth of microbes other than LAB, such as yeasts.

In previous research on sauerkraut, distinctive trends in redox potential were observed which followed the changes in organic acid and sugar content and followed the change from the initial fermentative microorganism *Leuconostoc mesenteroides* to *Lactobacillus plantarum*. (van Dijk and others 2000). Redox potential was also affected by the presence of *Enterobacter cloacae* (referred to as *Aerobacter cloacae*) in sauerkraut fermentation, and showed high reducing power versus the LAB normally present (Fulde and Fabian 1955).

The objective of this study was to observe changes in redox potential during pure culture pickle fermentations, in order to establish representative  $E_h$  trends for the environments

generated from growth of lactic acid bacteria and yeasts. Determination of such trends could aid in following the growth of beneficial or undesirable microorganism during cucumber fermentations.

### 2.3. Materials and Methods

#### 2.3.1. Culture Preparation

Cultures used in this research were obtained from the culture collection of the U. S. Department of Agriculture-Agricultural Research Service, Food Science Research Unit, located in Raleigh, North Carolina and manipulated as described on Table 2.1.

All culture media were obtained from Becton, Dickinson and Co. (Sparks, Md., U.S.A.).

**Table 2.1. Culture descriptions**

Microorganism	Culture Collection ID	Strain	Source	Culture Media	Incubation Temperature	Incubation Time
<i>Lactobacillus plantarum</i>	LA 0219	MOP3	Fermented cucumbers	MRS	30 °C	48 h
<i>Zygosaccharomyces globiformis</i>	Y0068, Y0069, Y0070, Y0071	SPY 9, 15, 21, 29	Spoiled sweet pickles	YM/A	30 °C	48 h
<i>Enterobacter aerogenes</i>	B0146	ATCC 13048	Bloated cucumbers	TSA	37 °C	24 h

#### 2.3.2. Cucumber Fermentation

Size 3B (39-51 mm diameter) cucumbers were collected from a local processor, rinsed in water and visually inspected. Any fruit with blemishes or deformities was discarded.

Cucumbers were diced into 10 mm cubes using a Hobart FP150 Food Processor (Hobart

Corporation, Troy, Ohio, U.S.A.). Diced cucumbers (1892 g) were covered with 1892 ml of cover solution in 3.79 liter glass jars (50:50 cucumber:brine pack out ratio). Cover brine solution contained acetic acid from commercial vinegar, sodium chloride (Morton Canning and Pickling Salt; Morton Salt, Chicago, Ill., U.S.A) and calcium chloride (Dow Chemical, Midland, Mich., U.S.A.) such that after equilibration with the cucumbers the final concentrations were 8.33 mM (0.05%), 1.03 M (6%) and 40 mM (0.44%), respectively. Jars were closed with commercial lug caps fitted with a rubber septum to allow for inoculation and sampling of the jars with sterile syringes and a metal fitting to hold a redox probe in place. The lids were heated in boiling water for at least 30 s to soften the sealing compound and immediately applied to the filled jars. Redox probes were calibrated, rinsed thoroughly with 70% ethanol and secured into the fittings on the jar lids. The tip of the probe was placed in the center of the jar and remained in contact with the brine cover solution.

The sealed jars were heated to a temperature of 74 °C for 15 min at the slowest heating point in the jars in a steam jacketed kettle and then cooled to 30 °C with cold tap water. This is a heat process typically used for pasteurization of commercial acidified vegetables. Triplicate jars were then inoculated with *L. plantarum* LA0219 ( $10^6$  CFU/ml), SPYs cocktail ( $10^3$  CFU/ml), *L. plantarum* LA0219 plus SPYs cocktail, *E. aerogenes* ( $10^6$  CFU/ml), *L. plantarum* LA0219 plus *E. aerogenes* and stored in a 30 °C incubator during fermentation. Six non-inoculated control jars were stored at 4 °C to prevent germination and growth of spores, which were not killed by the heat treatment. Samples for chemical and microbiological analyses and redox measurements were collected at the start of the

fermentations and at 24 hour intervals for a period of 12 days, in which fermentation proceeded to completion.

### **2.3.3. Evaluation of Microbial Growth**

Jars were visually monitored for the development of turbidity and/or pressure on the lids. Additionally, brine samples were collected using aseptic techniques for microbiological analysis. Samples were plated on deMan Rogosa and Sharpe agar (MRS) (Becton, Dickinson and Co.), Plate Count agar (PCA) (Becton, Dickinson and Co.), Yeast Mold agar (Becton, Dickinson and Co.) supplemented with 1 mM chlortetracycline (Sigma-Aldrich Chemical Co., St. Louis, Mo., U.S.A.) and 1.5 mM chloramphenicol (Sigma-Aldrich Chemical Co.) (YM/A), and Violet Red Bile agar (VRBA) (Becton, Dickinson and Co.). Plating was conducted using a spiral plater (Autoplate 4000, Spiral Biotech Inc., Norwood, Mass., U.S.A.). An automated colony counter (QCount, Spiral Biotech Inc.) was used to determine plate counts. MRS, YM/A, and PCA plates were incubated at 30 °C for 48 h. MRS plates containing a mixed microbial population were incubated under anaerobic conditions to prevent growth of yeasts. VRBA (Becton, Dickinson and Co.) plates were incubated aerobically at 37 °C for 24 h.

The occurrence of microbial growth was also evaluated by determination of changes in sugars, organic acids, and ethanol during incubation of samples at 30 °C. Brine and cucumber cube samples (50:50 w/v ratio) were collected daily using aseptic techniques and frozen at -80 °C. Prior to analysis, samples were thawed, macerated as required, and centrifuged at 12,000 rpm using an Eppendorf 5810R Centrifuge (Eppendorf North America,

Inc., Westbury, N.Y., U.S.A.). Analyses were done by HPLC on a 30 cm HPX-87H column (Bio-Rad Laboratories, Hercules, Calif., U.S.A.) (McFeeters and Barish 2003). The column was heated to 65 °C and eluted with 0.03 N sulfuric acid at a flow rate of 1.0 ml/min. Samples were analyzed for propionic acid and butyric acid using a UV6000 LP detector (Thermo Separation Products, Inc., San Jose, Calif., U.S.A.). A Waters model 410 refractive index detector (Waters, Milford, Mass., U.S.A.) connected in series was used to measure glucose, fructose, and ethanol.

#### **2.3.4. Determination of Redox Potential ( $E_h$ ) and pH**

Reference redox and pH measurements were taken every 24 h from each jar. pH was measured using an AccuFet solid-state pH combination electrode (Fisher Scientific, Pittsburgh, Pa., U.S.A.) and Accumet AR25 pH meter (Fisher Scientific) from aliquots of the equilibrated brine. Reference redox potential was measured using ORP combination electrodes (InLab 501/170, Mettler-Toledo, Bedford, Mass., U.S.A.) connected to a dual channel pH/ion meter (Accumet AR25, Fisher Scientific) to obtain readings using mV output. The ORP electrodes were calibrated using both pH 7 and pH 4 calibration buffers with added quinhydrone before each experiment. Alumina powder (1.0 micron; Precision Surfaces International, Houston, Tex.) was used to polish the metal tip of the electrodes periodically.

The  $E_h$  was calculated considering the changes on the reference redox potential in the presence of protons as described by the Nernst equation (1).

$$(1) \quad E_h = E'_{\text{ref}} + (RT/nF) \ln[H^+]$$

In this equation, R is the gas constant (J / K), T is the experimental temperature (K), F is the Faraday constant (C/mol), and n is the number of electrons involved in the redox reaction, which in the case of protons equals 1. The calculated  $E_h$  was also adjusted for the changes in pH during the fermentation (equation 2) since these changes are also detected by the ORP combination electrode. Equation (2) was modified as necessary for samples collected from jars incubated at 4 °C instead of 30 °C.

$$(2) \quad E_h = E'_{\text{ref}} - 0.0601 \times \text{pH}$$

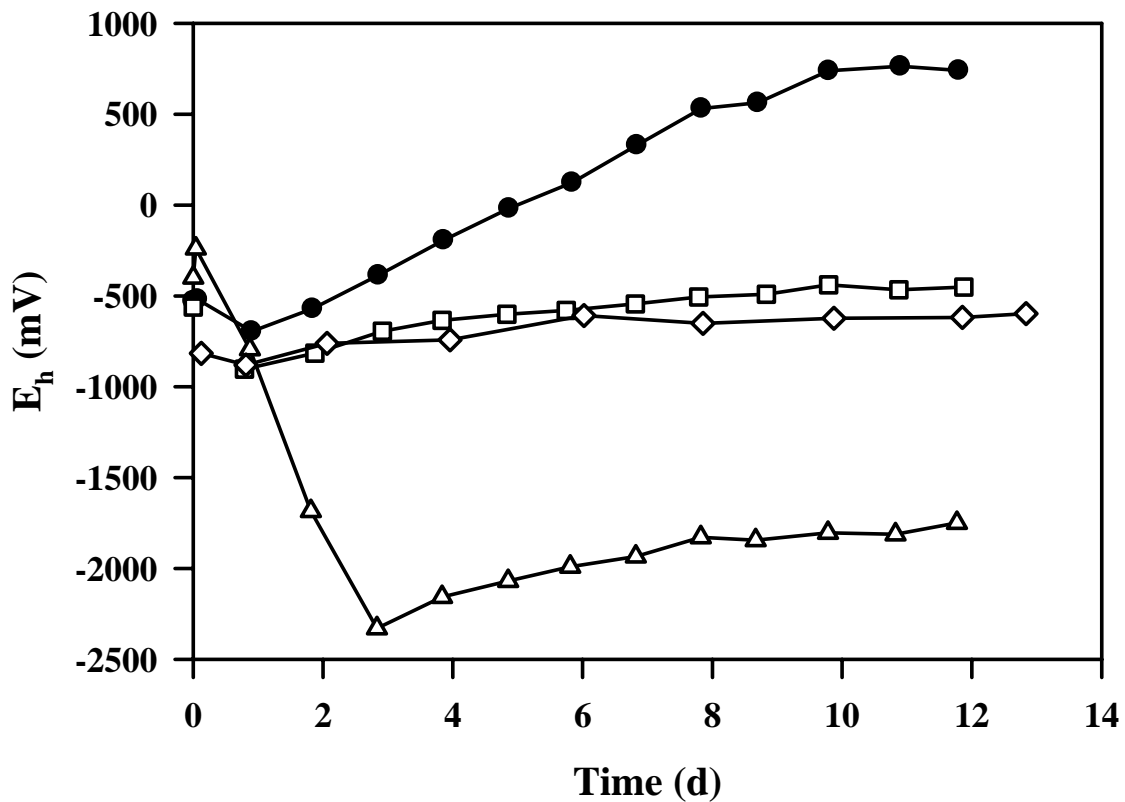
## 2.4. Results

Different trends in redox potential were observed in pasteurized cucumber jars inoculated with *L. plantarum*, *Z. globiformis*, and *E. aerogenes* (Fig. 2.1a). As expected, fermentation by *L. plantarum* induced an initial decline in redox potential due to the production of reducing compounds ( $73.96 \pm 12.2$  mM lactic acid) and consumption of oxidized molecules ( $45.68 \pm 6.2$  mM of hexose equivalents) (Fig. 1d) during metabolism (Ouvry and others 2001; Ouvry and others 2002; van Dijk and others 2000).  $E_h$  measurements unexpectedly increased as *L. plantarum* entered stationary phase (Fig. 2.1a and 2.1c).

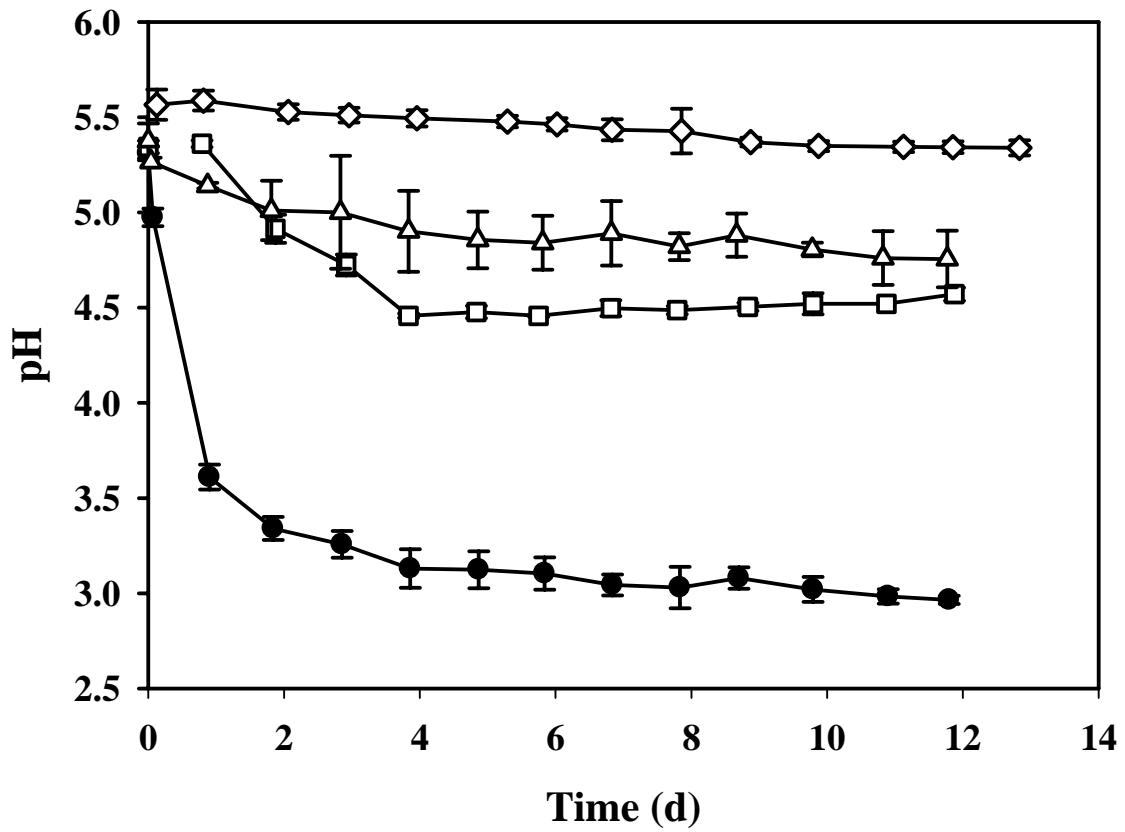
Similar to *L. plantarum*, growth of *Z. globiformis* caused an initial decline in the  $E_h$  trend (Fig. 2.1a and 2.1c). However, the  $E_h$  trend remained stable post-fermentation at a value similar to the  $E_h$  observed from non-inoculated jars (Fig. 2.1a). Unlike *L. plantarum*, *Z. globiformis* consumed  $38.45 \pm 1.9$  mM hexose equivalents (Fig. 2.1d) and produced  $49.62 \pm$

3.1 mM ethanol. As expected, growth of *E. aerogenes* caused a decline in  $E_h$ , which remained stable after the culture reached stationary phase (Fig. 2.1a and 2.1c).

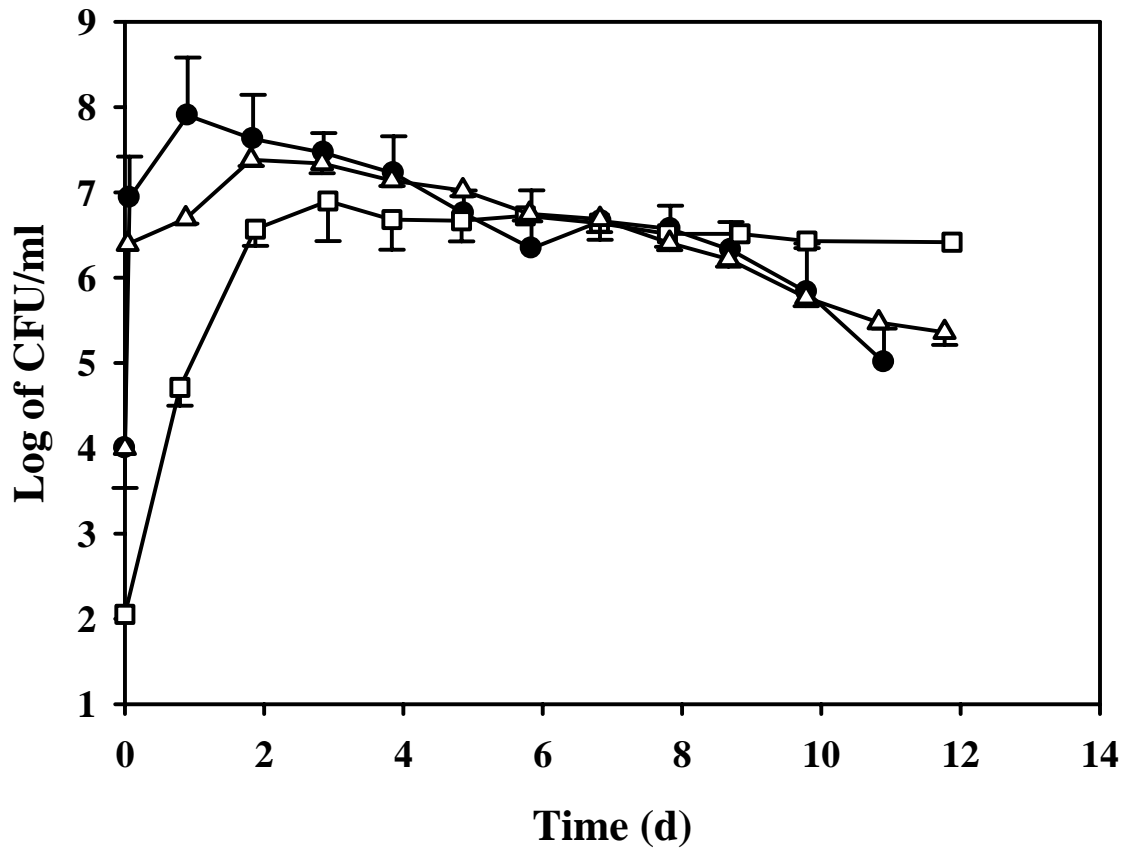
$E_h$  measurements indicated that jars inoculated with both *L. plantarum* and *Z. globiformis* (Fig. 2.2a) were more reduced than jars inoculated with only *L. plantarum* (Fig. 2.1a), and more oxidized than jars inoculated with only *Z. globiformis* (Fig. 2.1a), producing an  $E_h$  trend that lies in between the two individual trends (Fig. 2.2a). Although, the amount of lactic acid produced in this fermentation ( $76.96 \pm 10.52$  mM) was similar to the concentration obtained with *L. plantarum* alone, the amount of ethanol produced was 53% ( $22.66 \pm 6.3$  mM) of the final concentration in the *Z. globiformis* fermentation. However, the  $E_h$  trend from fermentation by a mixed culture of *L. plantarum* and *E. aerogenes* (Fig. 2.2a) was similar to the  $E_h$  trend of fermentation by *L. plantarum* (Fig. 2.1a).



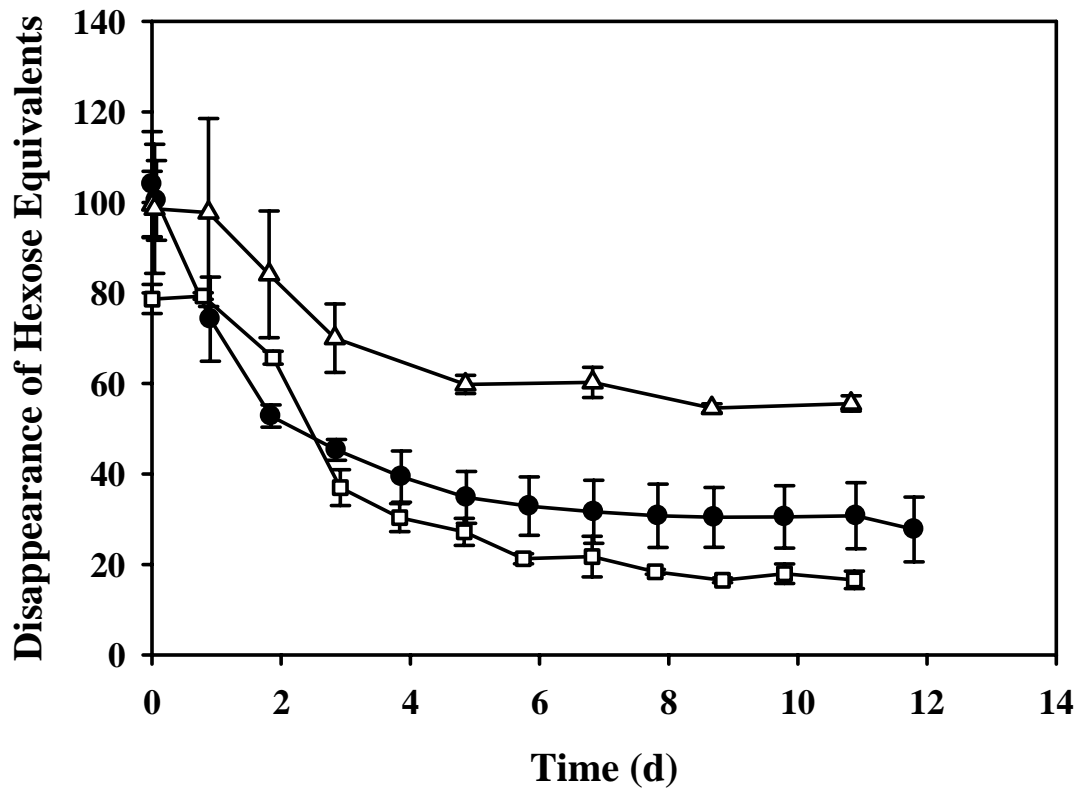
**Figure 2.1a. Influence of pure cultures on redox potential trends in pasteurized and brined cucumber jars.**  $E_h$  trends from cucumber jars inoculated with *L. plantarum* (●), *Z. globiformis* (□), and *E. aerogenes* (Δ), and non-inoculated control (◇).



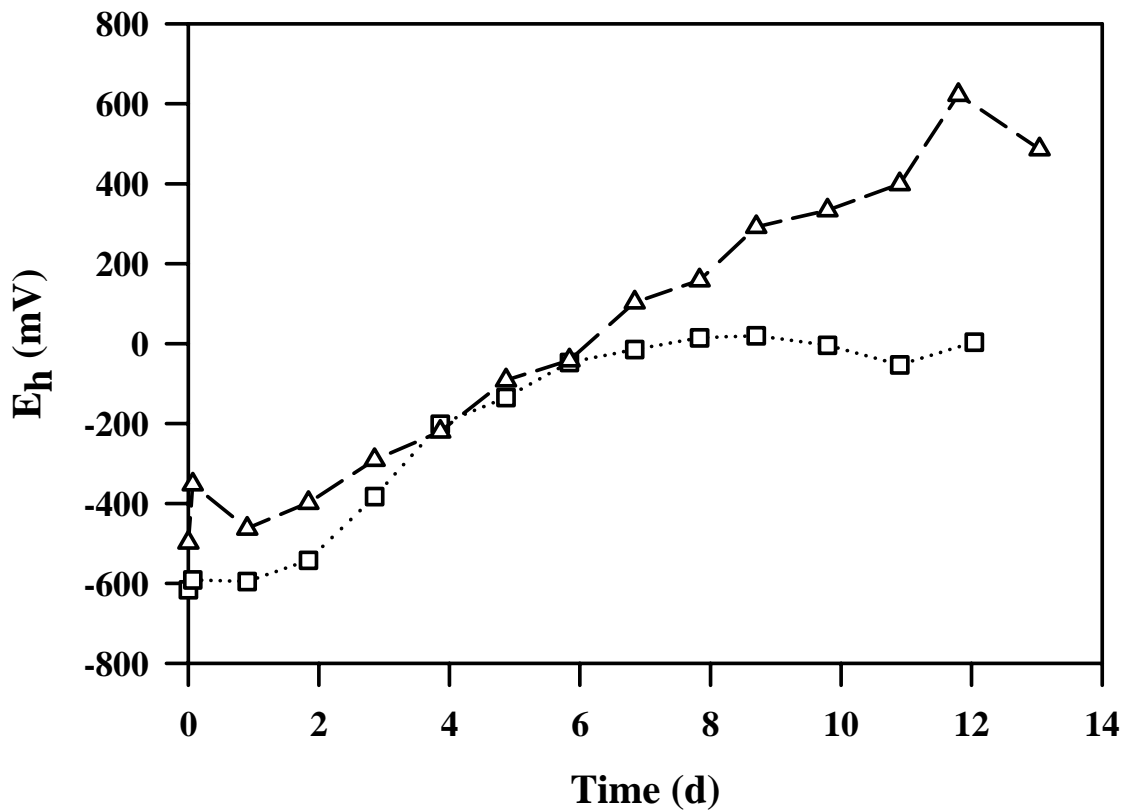
**Figure 2.1b. Influence of pure cultures on pH in pasteurized and brined cucumber jars.** Changes in pH from cucumber jars inoculated with *L. plantarum* (●), *Z. globiformis* (□), and *E. aerogenes* (Δ), and non-inoculated control (◇).



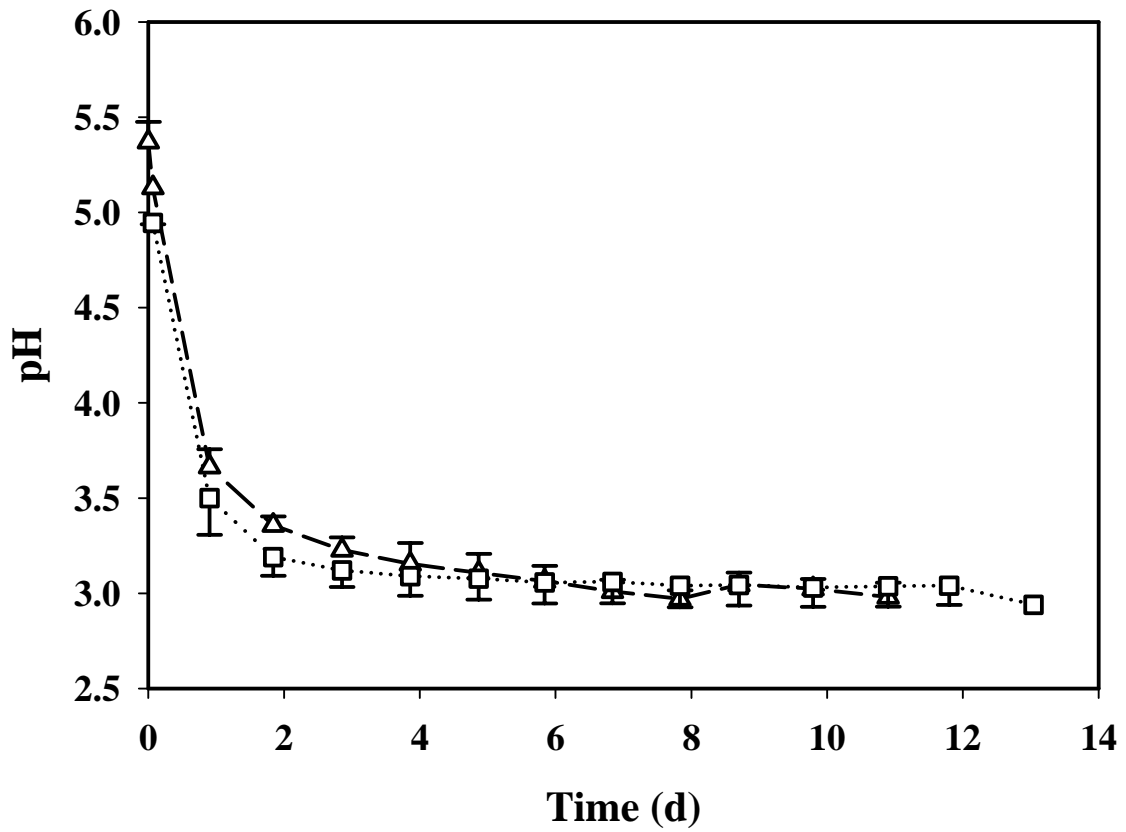
**Figure 2.1c. Microbial growth of pure cultures inoculated in pasteurized and brined cucumber jars.** Microbial growth monitored from cucumber jars inoculated with *L. plantarum* (●), *Z. globiformis* (□), and *E. aerogenes* (△), and non-inoculated control (◇).



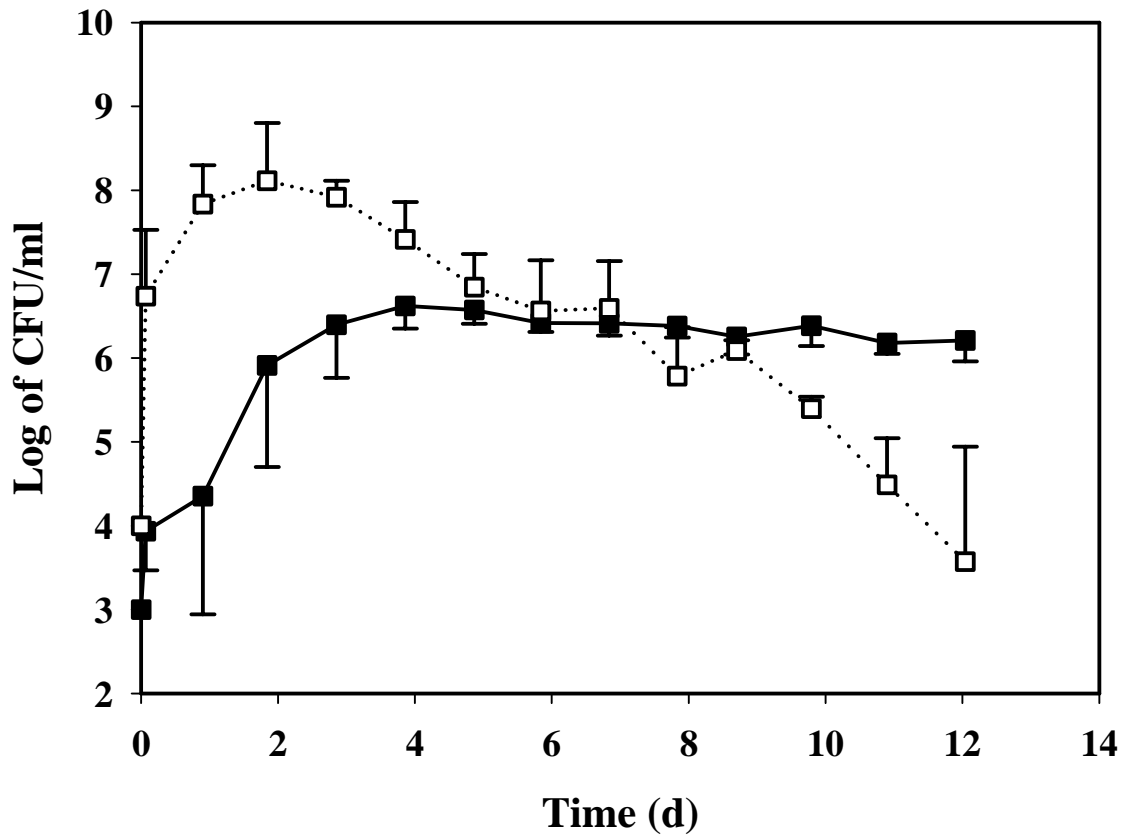
**Figure 2.1d. Influence of pure cultures on utilization of hexose in pasteurized and brined cucumber jars.** Disappearance of glucose and fructose, as represented by hexose equivalents, from cucumber jars inoculated with *L. plantarum* (●), *Z. globiformis* (□), and *E. aerogenes* (Δ), and non-inoculated control (◇).



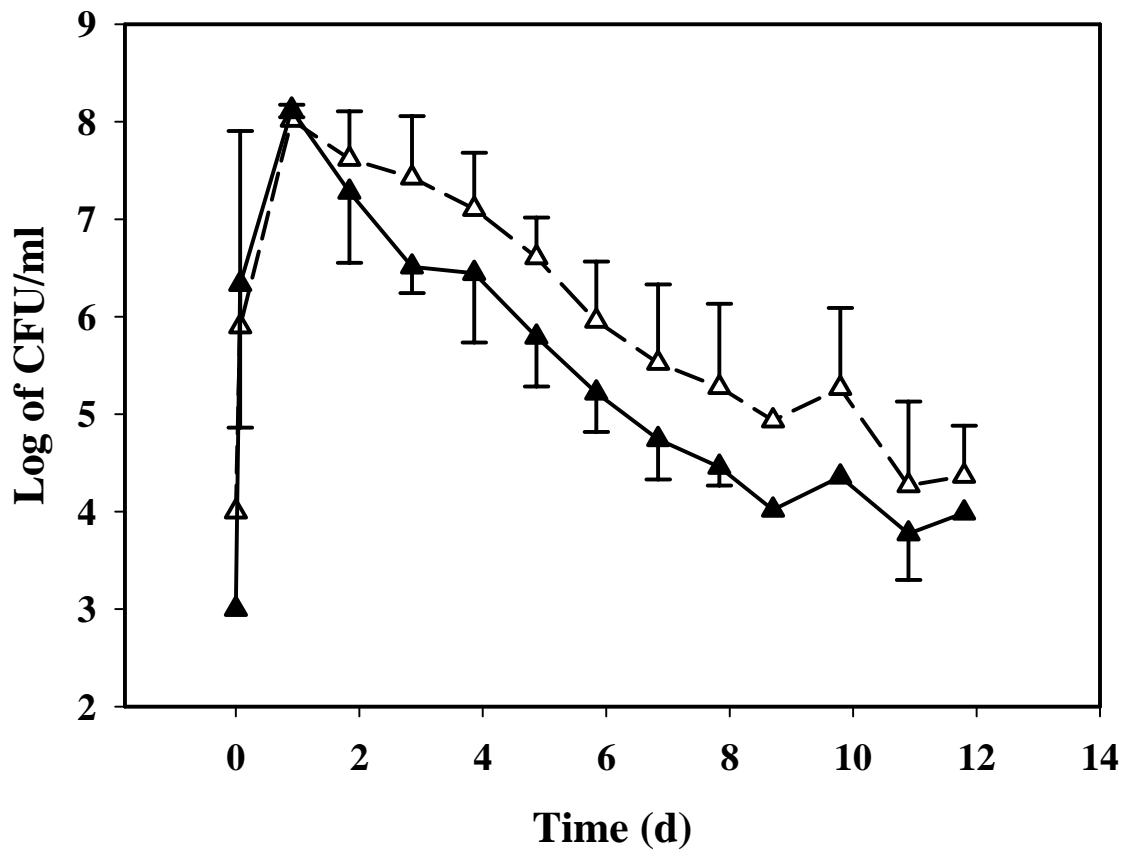
**Figure 2.2a. Influence of mixed cultures on redox potential trends in pasteurized and brined cucumber jars.**  $E_h$  trends from cucumber jars inoculated with *L. plantarum* and *Z. globiformis* ( $\square$ ), and *L. plantarum* and *E. aerogenes* ( $\Delta$ ).



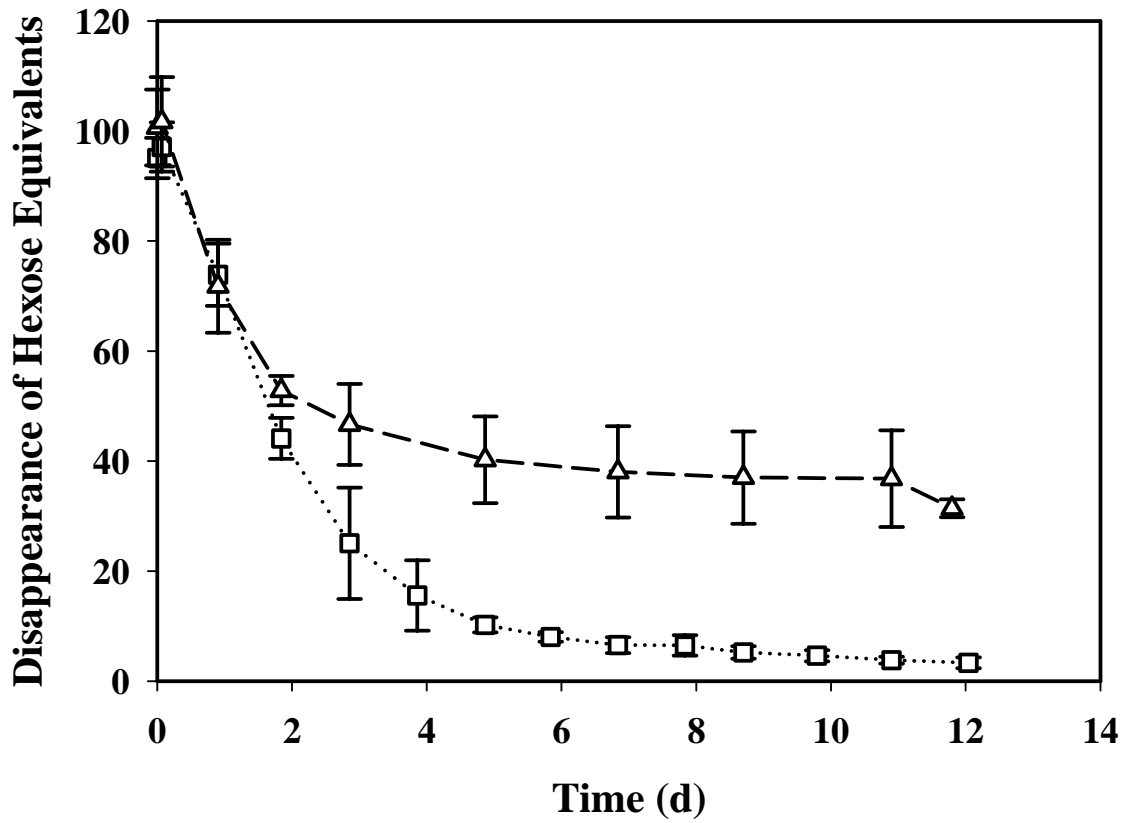
**Figure 2.2b. Influence of mixed cultures on pH in pasteurized and brined cucumber jars.** Changes in pH monitored in cucumber jars inoculated with *L. plantarum* and *Z. globiformis* (□), and *L. plantarum* and *E. aerogenes* (Δ).



**Figure 2.2c. Microbial growth from pasteurized and brined cucumber jars inoculated with a mixed culture of lactic acid bacteria and yeasts.** Microbial counts from cucumber jars inoculated with *L. plantarum* (□) and *Z. globiformis* (■).



**Figure 2.2d. Microbial growth from pasteurized and brined cucumber jars inoculated with a mixed culture of lactic acid bacteria and *Enterobacter aerogenes*. Microbial counts from cucumber jars inoculated with *L. plantarum* ( $\Delta$ ) and *E. aerogenes* ( $\blacktriangle$ ).**



**Figure 2.2e. Influence of mixed cultures on utilization of hexose in pasteurized and brined cucumber jars.** Disappearance of glucose and fructose, as represented by hexose equivalents, from cucumber jars inoculated with *L. plantarum* and *Z. globiformis* (□), and *L. plantarum* and *E. aerogenes* (Δ).

## 2.5. Discussion

Commercial cucumber fermentations are typically predominated by growth of acid resistant lactic acid bacteria, primarily *Lactobacillus plantarum*. However, the natural cucumber microflora may vary with the production geographic area, climate, and soil conditions, the harvesting season, and distribution channels. As a result, atypical fermentations occasionally occur with undesirable consequences. The approach taken here was to remove the naturally occurring vegetative cells of microorganism from the cucumbers, inoculate the brined cucumber cubes with known cultures of bacteria or yeasts, and then observe changes in  $E_h$  along with measurements of pH and fermentation substrates and products. In this way  $E_h$  was correlated with parameters that have been previously used to follow the course of cucumber fermentations.

Overall, the observed trends from microbial growth, pH, availability of hexose equivalents, and production of lactic acid or ethanol was not parallel to the changes observed in the  $E_h$  trends. Fluctuations in the concentrations of the fermentation substrates such as glucose and fructose (Fig. 2.1d and 2.2e) or in metabolic end products such as ethanol and lactic acid did not directly influence the observed  $E_h$  trends, which reflects the more comprehensive nature of this variable. However, a steady increase in the  $E_h$  measurements from the fermentations in which *L. plantarum* predominated was observed while the number of viable cells of this bacterium diminished. Presumably, the steady increase in the  $E_h$  trend was caused by induction of *L. plantarum* autolysis (Gasson 1996; Weinrichter and others 2003) or viable but not culturable state (Oliver 2005), and/or by sporulation of the oxidizing bacteria, *Bacillus*

spp. (Rödel and Lücke 1990). Certainly, further research is required to elucidate the cause for the increase in  $E_h$  post-fermentation. The increment in  $E_h$  was still observed after the corresponding pH trends reached a plateau (Fig. 2.1a and b and 2.2a and b) and the hexose equivalents were fully depleted. The data suggests that the  $E_h$  trend would provide a more in-depth view of the chemical changes occurring during desirable cucumber fermentations by *L. plantarum*. A lack of an increment in the  $E_h$  measurements after completion of the fermentation, as indicated by a stable pH and exhaustion of the hexose equivalents, could serve as an on-site and immediate indicator of growth of undesired yeasts during storage.

*E. aerogenes* generates hydrogen gas as a metabolic end product, which is a strong reductant capable of creating a highly reduced environment (Johansen and others 1975). Lack of fitness of *E. aerogenes* to the cucumber fermentation environment is reflected in the fact that only 63.4 % ( $29.18 \pm 1.3$  mM hexose equivalents) of the carbohydrates available was utilized (Fig. 2.1c).

The increase in  $E_h$  measurements (Fig. 2.2a) and the decrease in pH (Fig. 2.2b) in the presence of a mixed culture with *L. plantarum* and *E. aerogenes* is a mere reflection of the dominant role of *L. plantarum* during a fermentation, in which the emerging acidic conditions preclude the survival of *E. aerogenes* (Etchells and Jones 1943).

## **2.6. Conclusions**

Distinctive trends in redox potential during fermentation of brined, pasteurized and inoculated cucumber jars were observed, and depended upon the microbial background.

Trends in pH with time were not as variable as the observed  $E_h$  patterns. Thus, it should be possible to utilize  $E_h$  measurements as a tool to monitor microbial growth and the progress of natural cucumber fermentations in the field. Such  $E_h$  monitoring could provide information, regarding growth of undesired yeasts, which would not be obvious from pH monitoring alone.

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## **Chapter 3.**

### **Preliminary Evaluation of Redox Potential Trends in Non-Pasteurized Jars of Fermented Cucumbers**

### 3.1. Abstract

pH measurements have been used for decades to monitor the progression of cucumber fermentations in the field. Although, pH measurements indirectly reflect growth of lactic acid bacteria and acid production, it is inadequate for the detection of undesired yeast growth. Alternatively, redox potential ( $E_h$ ) measurements have the potential to describe the fermentation environment more globally and inclusively. Natural cucumber fermentation and corresponding redox potential ( $E_h$ ) measurements were examined in fermented cucumbers to establish trends that could serve as a basis for the application of this tool. Jars of various sized cucumbers were packed and brined in ways that allowed for a natural microbial succession or preservation to proceed. Under each unique instance, a different trend in  $E_h$  was observed. Steady plateaus in  $E_h$  were observed in the absence of microbial growth, independent of cucumber sizes.  $E_h$  values initially decreased as natural lactic acid bacteria or inoculated *Lactobacillus plantarum* grew, but increased following cell death. Jars in which spoilage yeast naturally occurred maintained a consistently lower  $E_h$  trend than jars in which lactic acid bacteria predominated suggesting that redox potential is indeed a tool capable of differentiating between these two events. By measuring  $E_h$ , it may be possible to predict yeast spoilage earlier than by pH or visual analysis. Extension of redox potential applications into other fermentation products may prove valuable as well.

### 3.2. Introduction

Numerous food and beverage products around the world are preserved through the centuries old process of fermentation to extend shelf-life and gain distinctive flavor characteristics.

Fermented vegetable products, including pickles, are one of the popular commodities.

Pickles are made by the fermentation or acidification of cucumbers, and are available in an assortment of varieties. Lactic acid bacteria (LAB) are indigenously present in cucumbers, and are a necessary component of natural cucumber fermentation. LAB produce lactic acid through the fermentation of available carbohydrates, which in turn lowers the pH of their environment. The low pH is often inhospitable for other microorganisms that may be present on the fruits (Breidt 2006). Some yeasts are able to tolerate the harsh conditions created by the LAB, but are an undesirable microbe for pickle production. Rather than lactic acid fermentation, yeasts undergo ethanolic fermentation, creating byproducts not suitable in pickle products such as ethanol and carbon dioxide (White 2007). The presence of ethanol would create an undesired flavor profile and carbon dioxide production could lead to bloater pickles which have gas pockets inside the cucumber flesh.

While pH is commonly used to monitor fermentation, redox potential ( $E_h$ ) may be an alternate prospective tool. Redox potential is the tendency of a chemical substance to gain or lose electrons. Electrons are gained during reduction reactions and are donated during oxidation reactions. During the transfer of electrons in oxidation-reduction reactions, energy is created for microbial cells (Prescott and others 2005). Since redox potential evaluates the activity of electrons present, rather than solely measuring the concentration of hydrogen ions like pH, it may be a more extensive tool to monitor changes during fermentation (Kjaergaard 1977).

The purpose of this research was to compare redox potential trends with various sizes of pickling cucumbers and to establish general  $E_h$  trends for cucumbers undergoing natural fermentation. Cucumbers can be differentiated by their diameter and length, the smallest being 1A and largest being 3B. Smaller cucumbers have shown to have less fermentable sugars available and also have a higher buffering capacity (Lu and others 2002). Jars of various sized cucumbers were brined and continuously evaluated for changes in  $E_h$  in the presence of microbial growth.

### **3.3. Materials and Methods**

#### **3.3.1. Culture Preparation**

Pure cultures of *Lactobacillus plantarum* LA0219 were obtained from the culture collection of the U. S. Department of Agriculture-Agricultural Research Service, Food Science Research Unit, located in Raleigh, North Carolina. The strain had been previously isolated from cucumber fermentations. *L. plantarum* LA0219 was incubated under aerobic conditions at 30 °C for 48 h using deMan, Rogosa, and Sharpe agar (MRS) (Becton, Dickinson and Co., Sparks, Md., U.S.A.).

#### **3.3.2. Cucumber Jars**

Cucumbers with varying diameter (1A = less than 19 mm, 1B = 20 to 25 mm, 2B = 32 to 38 mm, 3B = 44 to 51 mm) were acquired from a local processor, rinsed in water and examined visually. Soiled or damaged cucumbers were removed. Whole cucumbers (1892 g) were packed in 3.79 L glass jars and covered with 1892 ml of cover solution (50:50

cucumber:brine pack out ratio). Equilibrated brine for the jars contained 1.59% (26.5 mM) acetic acid, 0.22% (20 mM) calcium chloride and 6.65% (1.15 M) sodium chloride (Morton Canning and Pickling Salt; Morton Salt, Chicago, Ill., U.S.A.). Commercial lug caps were made to include both a rubber septum to allow for inoculation and sampling of the jars with sterile syringes and a metal fitting to secure a redox electrode. Lids were boiled in water for 30 s and firmly hand twisted onto the jars. Redox probes were calibrated using solutions of pH 7 and pH 4 buffers plus quinhydrone. The probes were sanitized using 70% ethanol and inserted into the metal fittings. The tip of the probe was positioned in the middle of the jar to get an air-free, accurate  $E_h$  measurement. A silicone sealant was applied around the fittings holding the redox probes for jars in which no yeast growth was desired. Each trial was completed in triplicate. All jars were stored at room temperature (24 to 27 °C). Following jar equilibration, cover brine solution was sampled every 12 or 24 h for chemical and microbiological analyses.

### **3.3.3. Evaluation of Redox Potential and Microbial Growth**

Daily redox and pH measurements were recorded for each jar trial. Jars were also examined for any visually noticeable physical changes, such as turbidity, pressure on the lids, gas bubble formation, and film yeast growth. An AccuFet solid-state pH combination electrode (Fisher Scientific, Pittsburgh, Pa., U.S.A.) and Accumet AR25 pH meter (Fisher Scientific) were used to measure pH. ORP combination electrodes (InLab 501/170, Mettler-Toledo, Bedford, Mass., U.S.A.) were used in each jar to measure the changes in redox potential. Millivolt measurements were taken by connecting the ORP probes to a dual channel pH/ion

meter (Accumet AR25, Fisher Scientific). The measured redox potential was converted to  $E_h$  using the manufacturers instructions ( $E_h = E_{meas} + 207 \text{ mV}$ , at  $25 \text{ }^\circ\text{C}$ ). The metal tip of the probes were polished using an alumina powder (1.0 micron; Precision Surfaces International, Houston, Tex.) mixture when necessary.

The reference redox potential ( $E_{ref}$ ) measured by the ORP electrodes was standardized to the standard hydrogen electrode ( $E_h$ ) using the Nernst equation:

$$E_h = E_{ref} + (RT / nF) \ln[H^+]$$

Where  $R$  is the gas constant ( $\text{J K}^{-1}$ ),  $T$  is the absolute temperature (K),  $n$  is the number of electrons involved ( $n = 1$ , for protons)  $F$  is the Faraday constant ( $\text{C mol}^{-1}$ ).

The simplified equation allows for normalization of the redox reading according to pH change, at  $25 \text{ }^\circ\text{C}$ :

$$E_h = E_{ref} - 0.05913 \times pH$$

Samples of cucumber brine were aseptically removed daily to monitor microbial growth. Brine was plated for LAB counts using deMan Rogosa and Sharpe agar (MRS) (Becton, Dickinson and Co.), and plated for yeasts using Yeast Mold agar (Becton, Dickinson and Co.) supplemented with 1 mM chlortetracycline (Sigma-Aldrich Chemical Co., St. Louis, Mo., U.S.A.) and 1.5 mM chloramphenicol (Sigma-Aldrich Chemical Co.) (YM/A). Both MRS and YM/A plates were incubated at  $30 \text{ }^\circ\text{C}$  for 48 h. YM/A plates were incubated under aerobic conditions and MRS plates were incubated anaerobically to prevent growth of yeasts. A spiral plater (Autoplate 4000, Spiral Biotech Inc., Norwood, Mass., U.S.A.) and an

automated colony counter (QCount, Spiral Biotech Inc.) were used to enumerate microbial counts.

Additional samples of brine were aseptically collected daily and maintained at -80 °C.

Samples were thawed and centrifuged at 6,000 rpm using an Eppendorf 5810R Centrifuge

(Eppendorf North America, Inc., Westbury, N.Y., U.S.A.) before chemical analysis. The

production and utilization of sugars, organic acids, and ethanol during the course of the

fermentation were analyzed by HPLC using a 30 cm HPX-87H column (Bio-Rad

Laboratories, Hercules, Calif., U.S.A.) (McFeeters and Barish 2003). The column was

heated to 65 °C and eluted with 0.03 N sulfuric acid using a flow rate of 1.0 ml/min.

Analysis of organic acids was conducted using a UV6000 LP detector (Thermo Separation

Products, Inc., San Jose, Calif., U.S.A.). Sugars, propionic acid, and ethanol were measured

by a Waters model 410 refractive index detector (Waters, Milford, Mass., U.S.A.).

### **3.4. Results**

Varying trends in redox potential were observed in non-pasteurized jars of brined cucumbers.

Correlating changes in pH and microbial counts were also observed. It was found that a

difference in cucumber size (1A, 2B, or 3B) had a minimal impact on the resulting  $E_h$  trend

in the absence of microbial growth. A distinction between the presence of lactic acid bacteria

or spoilage yeasts was evident in the resulting measured redox potential during the

fermentation.

### **E<sub>h</sub> Trends from Cucumbers of Various Sizes**

The trends in redox potential for non-pasteurized jars of brined cucumbers of assorted sizes are shown in Figure 3.1. Size 1A, 2B, and 3B cucumbers were stored over 16 d without any microbial growth. Beginning at a high, positive initial redox potential ( $E_h = +599.5$  mV, size 3B; +667.3 mV, 1A; +926.4 mV, 2B), the  $E_h$  trend rapidly declined within the first 2 d for each series of jars, reaching and maintaining a range of +100 to -300 mV. The overall  $E_h$  trend was observed between each of the sizes; however, some deviations in measurements were seen. The size 3B cucumbers sustained an  $E_h$  slightly more oxidized than the 2B's and the 1A's.

### **E<sub>h</sub> Trends During Natural Cucumber Fermentation**

A distinct correlation between redox potential, pH, and microbial counts (Figure 3.2 and 3.3) was observed during lactic acid bacteria fermentation. An initial decrease in  $E_h$  developed within the first 48 h (+599.8 mV, 0 d to -133.5 mV, 2d), followed by a 5 d plateau where the  $E_h$  remained constant. Another sharp decline in  $E_h$  was observed after 12 d ( $-830.5 \pm 89.2$  mV), along with a 4 log increase in LAB counts and decrease in pH from 4.1 ( $\pm 0.06$ ) to 3.6 ( $\pm 0.31$ ). The  $E_h$  increased at the end of the fermentation (roughly -100 mV to -300 mV), as the counts of LAB began to decrease and have less impact on controlling the  $E_h$  trend.

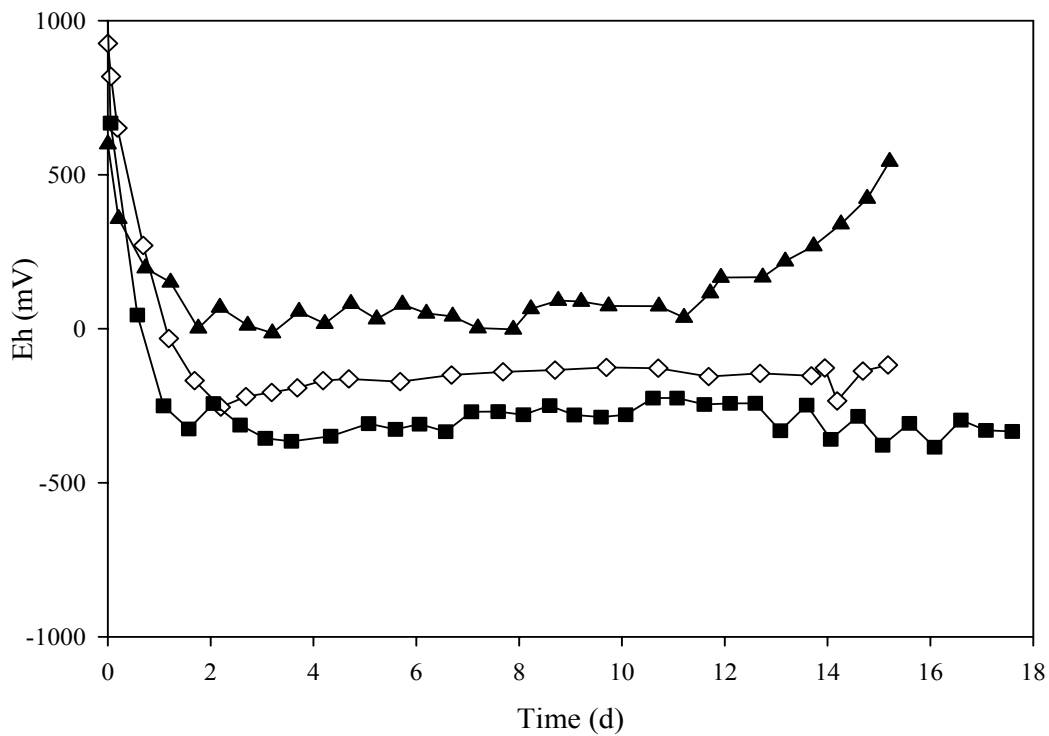
The natural fermentation proceeded to completion as reflected by full utilization of the carbohydrates available ( $32.6 \pm 1.17$  mM hexose equivalents). Lactic acid ( $64.9 \pm 4.75$  mM)

was the primary product during the natural fermentation. As expected only basal levels of ethanol were detected ( $6.84 \pm 6.37$  mM).

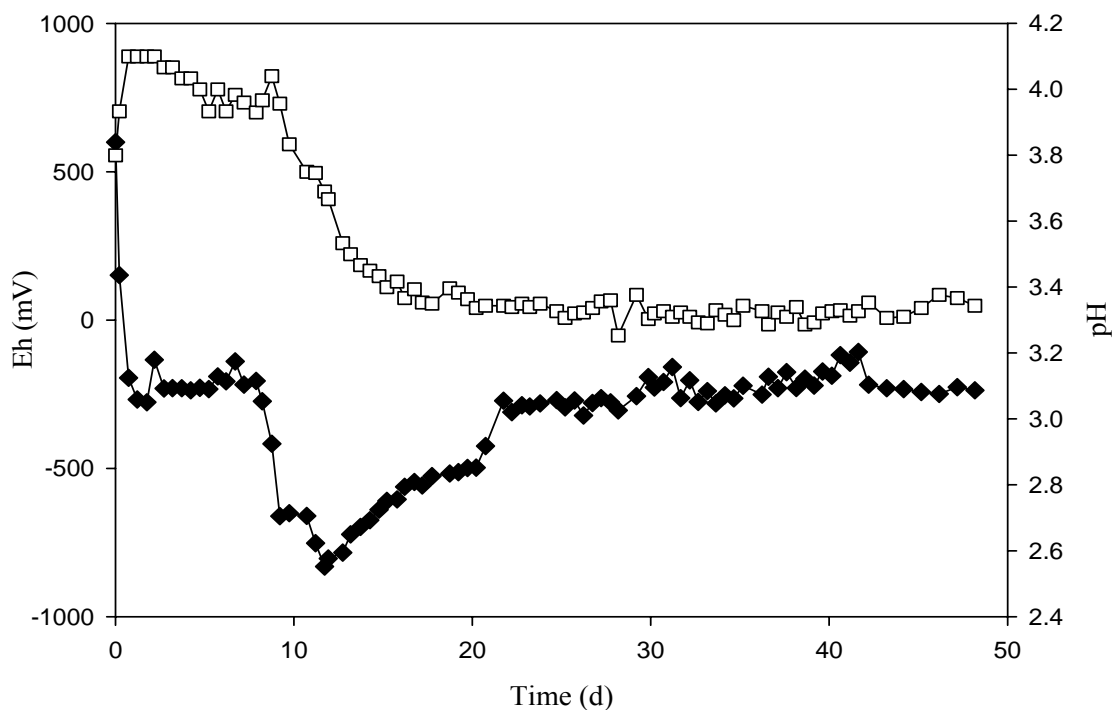
### **$E_h$ Trends During Natural Cucumber Fermentation in Which Film Yeast Growth Developed**

The impact that natural yeast spoilage of cucumbers had on trends on redox potential were examined (Figure 3.4). Towards the end of the fermentation measured pH varied slightly ranging from ~3.5 to 3.8.  $E_h$  began at  $+882.5 (\pm 53.5)$  mV, fell to  $+152.1 (\pm 25.1)$  mV after 3 d, and entered a slow decline, with a final measured  $E_h$  of  $-229.6 (\pm 145.3)$  mV after 36 d. Growth of film yeasts in the headspace of the jars was noted after 3 d. YM/A counts reached  $10^4$  CFU/ml by 6 d and  $10^7$  CFU/ml by 24 d (Figure 3.5).

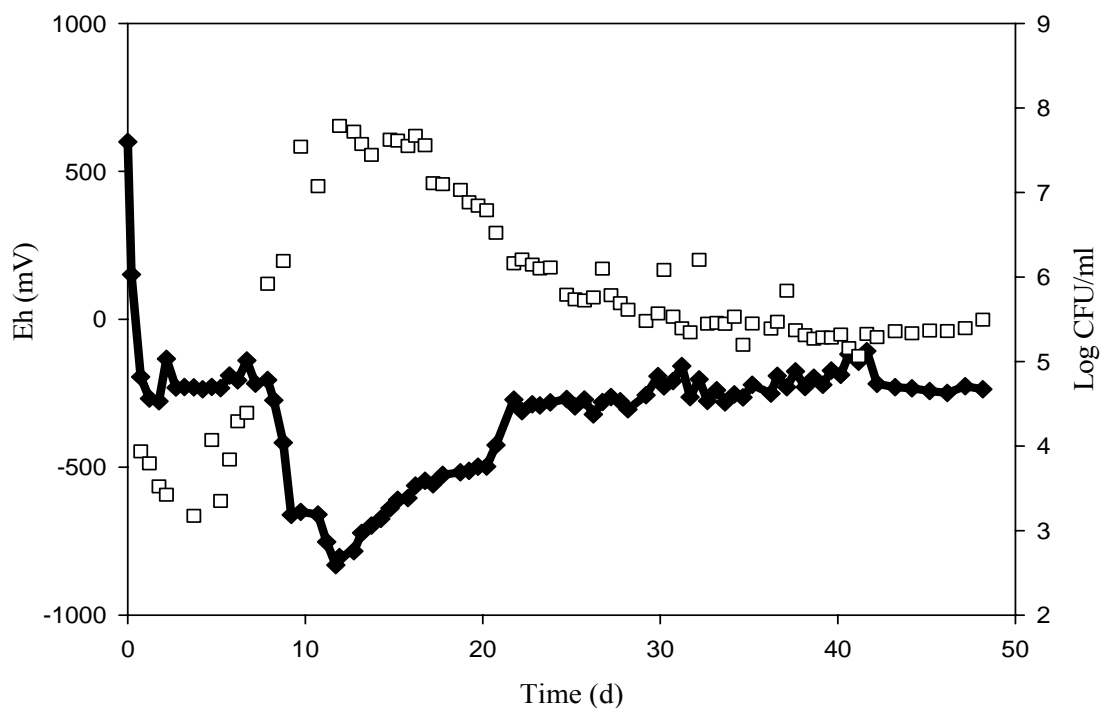
The natural fermentation in which film yeast growth developed proceeded to completion. A total of  $29.2 \pm 0.68$  mM hexose equivalents were converted into  $42.7 \pm 27.2$  mM lactic acid and  $25.3 \pm 7.5$  mM ethanol.



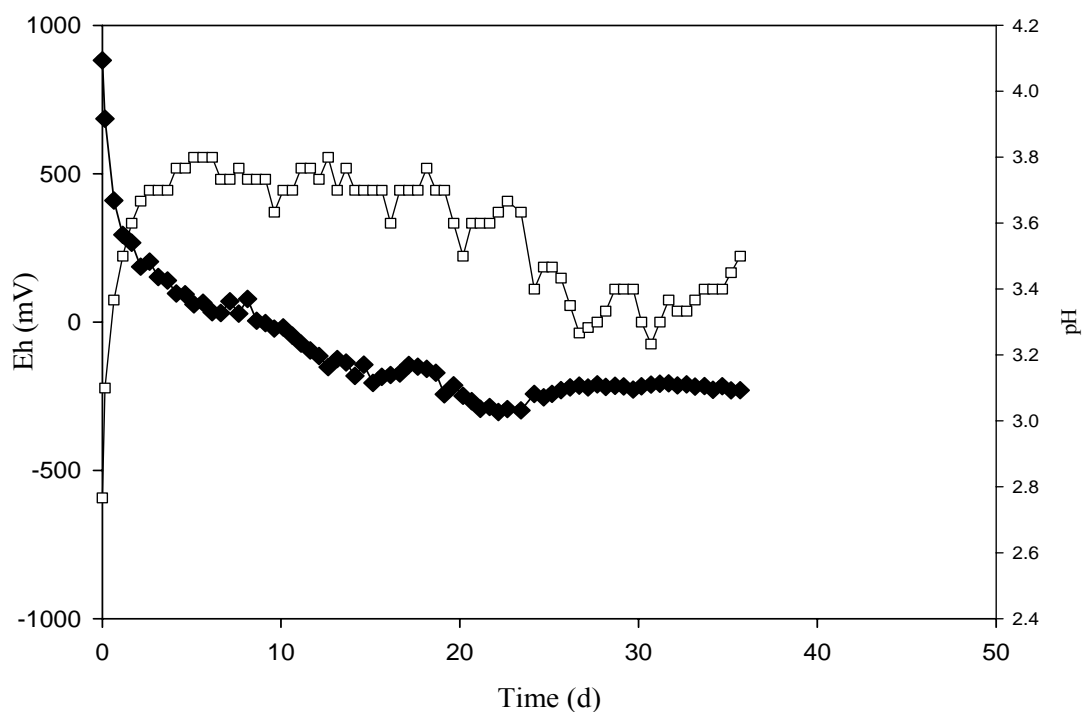
**Figure 3.1. Average trend of redox potential between various sizes of cucumbers: 1A vs. 2B vs. 3B.** Redox potential trends for 2B cucumbers ( $\diamond$ ), 1A's ( $\blacksquare$ ), and 3B's ( $\blacktriangle$ ) show minimal variation over time. Maximum standard deviations calculated were  $\pm 253.8$  mV (0 to 2 d),  $\pm 157.6$  mV (2 to 14 d), and  $\pm 249.0$  mV (14 to 18 d).



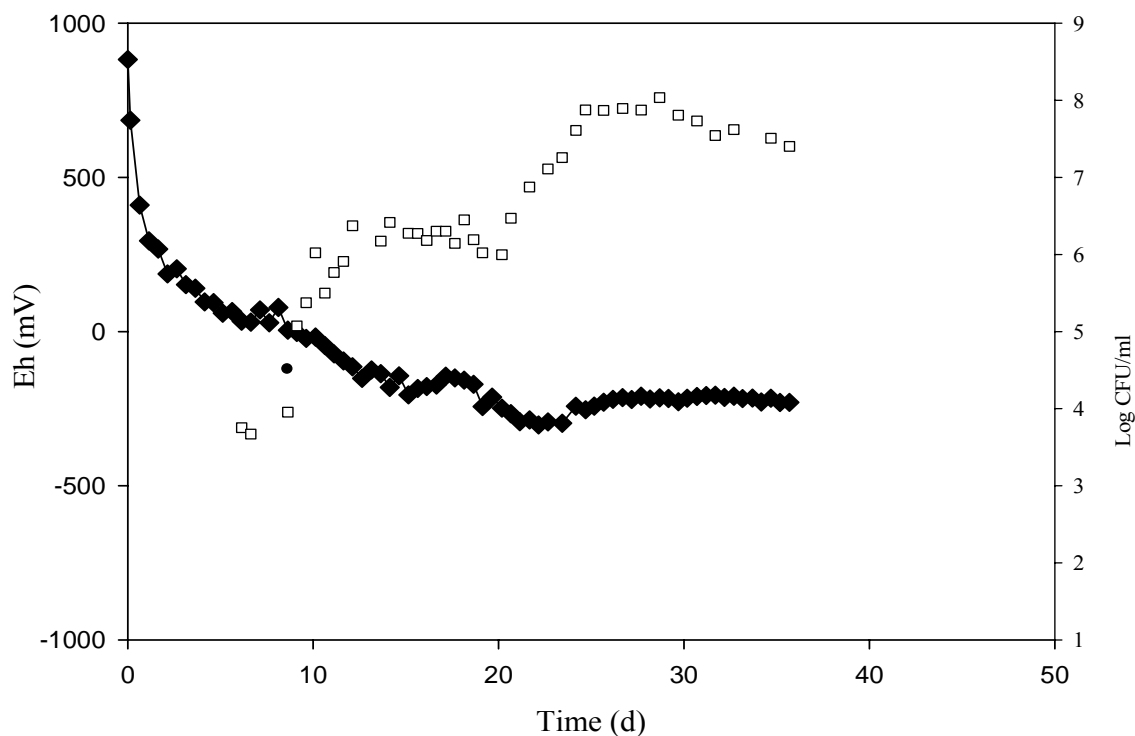
**Figure 3.2. Average trend of redox potential and pH in cucumbers jars during natural fermentation by lactic acid bacteria.** Redox potential (◆) sharply decreases with a drop in pH (□). Maximum standard deviations calculated were  $\pm 475.3$  mV, and  $\pm 0.31$  pH units.



**Figure 3.3. Average trend of redox potential and microbial counts in cucumbers during natural fermentation by lactic acid bacteria.** Redox potential (♦) declines as counts of LAB increase (□). Maximum standard deviations calculated were  $\pm 475.3$  mV, and  $\pm 1.31$  log of CFU/ml.



**Figure 3.4. Average trend of redox potential and pH in fermented cucumber jars in which yeast spoilage developed.** Redox potential (♦) and pH (□) trends slightly decline and remain constant over time in the presence of spoilage yeasts. Maximum standard deviations calculated were  $\pm 151.7$  mV, and  $\pm 0.58$  pH units.



**Figure 3.5. Average trend of redox potential and microbial counts in fermented cucumber jars in which yeast spoilage developed.** Redox potential (◆) does not increase following the growth of spoilage yeasts (□) in the jars. Maximum standard deviations calculated were  $\pm 151.7$  mV, and  $\pm 1.17$  log of CFU/ml.

### 3.5. Discussion

The natural presence and growth of yeasts during cucumber fermentation is an unwanted and reoccurring problem in the pickling industry. Spoilage yeasts are often able to tolerate the conditions of fermentation and go undetectable by pH monitoring. The use of continuous  $E_h$  measurements during the fermentation process has the potential to be a way to evaluate if substantial yeast growth occurs. The focus of this research was to evaluate redox potential as

a tool to differentiate between the growth of lactic acid bacteria and growth of yeast spoilage organisms in fermented cucumbers.

Cucumber compositions are slightly different, with larger 3B's generally containing more fermentable sugars than smaller cucumbers (Lu and others 2002), accounting for some variability of  $E_h$  between sizes. These differences did not significantly influence the overall redox potential trends with the exception of a longer equilibration phase, and a slightly more oxidized stable final  $E_h$  for the largest fruits.

An initial decrease in  $E_h$  was observed in fermented cucumber jars. However, a further sharp decline occurred in the absence of yeast spoilage.  $E_h$  and pH values steeply declined with an increase in growth of lactic acid bacteria throughout the fermentation. The role of LAB in  $E_h$  trends was reflected in the fact that when metabolized sugars were converted to reduced components the redox potential trend changed towards more reduced values (Ouvry and others 2001; van Dijk and others 2000) until it reached -830.5 mV. When yeast spoilage proceeded, a more oxidized and steady  $E_h$  trend was established at +267.4 mV, which significantly differed from the drastic decline observed from cucumbers under natural LAB fermentation. Previous research on redox potential in pasteurized cucumber jars has also shown that the presence of lactic acid bacteria and spoilage yeasts are distinguishable by changes in  $E_h$  trends (Chapter 2). The compounds produced by spoilage yeast and LAB during fermentation are different, which works to maintain distinctly unique overall trends in  $E_h$ .

Further exploration of measuring  $E_h$  in the field may prove to be beneficial to the industry. If a large sample of cucumber fermentation tanks were monitored by  $E_h$ , a more in depth understanding of this technology can be evaluated and beneficially applied commercially. As a better comprehension of large scale cucumber fermentation processes is established, this research can then extend into other fermented food and beverage avenues.

### **3.6. Conclusions**

Unique trends in redox potential were observed for non-pasteurized jars of various sized cucumbers under a range of conditions. When natural LAB fermentation proceeded, a sharp decline in  $E_h$  was consistently measured. This could be differentiated from jars with yeast spoilage, as distinct redox potential trends were noted in either case. The implementation of measuring redox potential may be useful for the fermentation industry to distinguish between desirable or undesirable microbial conditions.

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## **Chapter 4.**

### **Influence of Gas Purging on Microbial Growth and Redox Potential Trends in Fermented Cucumbers**

#### **4.1. Abstract**

Cucumber fermentation tanks are commonly purged to prevent cucumber bloating due to the production of carbon dioxide. Although this practice has been applied for years, the influence of gas purging in the development of the fermentation as reflected on acid production, microbial growth, pH, and redox potential ( $E_h$ ) has not been studied. Brined cucumbers were packed into 2 L fermentor systems and initially sparged with nitrogen, oxygen, hydrogen, or remained without treatment.  $E_h$ , pH, microbial counts, organic acids and carbohydrates were monitored throughout the fermentations. As expected, significant changes in  $E_h$  measurements were observed and varied according to the gas that was sparged into the system. Minimal differences were detected in pH and total counts of lactic acid bacteria, and carbohydrate utilization, suggesting that the experimental gas sparging did not influence the development of the fermentation. However, the LAB growth rate and subsequent lactic acid production were the most effective under nitrogen sparging. Also, significantly higher yeasts counts and growth rate were measured when oxygen purging was applied.

#### **4.2. Introduction**

The ancient art of fermentation is a practice that has been cultivated worldwide to preserve and to create desired food and beverage items. Vegetables, including cucumbers, are often fermented by the succession of lactic acid bacteria (LAB), the predominant microflora. Under the necessary pickling conditions, these bacteria utilize available carbohydrates, produce lactic acid, and lower the surrounding pH, thus working to preserve the product and

establish desired flavor characteristics (Breidt and others 2007). *Lactobacillus plantarum* predominates in cucumber fermentation, due to its ability to withstand low pH and high salt conditions (Fleming and McFeeters 1981; Hutkins 2006). The progression of the fermentation in the field is frequently determined by monitoring changes in pH. Measuring redox potential ( $E_h$ ) is an alternate technique to monitor variations during fermentation.

Redox potential is a measure of the electron flow during chemical reactions as oxidation and reduction reactions occur. As a system is oxidized, characterized by positive mV readings, electrons are lost. During reduction, electrons are gained, and can be indicated by negative mV readings (Lehninger and others 1993). When redox potential is measured, the activity of the electrons in the system is taken into account. Monitoring  $E_h$  may offer more value than pH, which only measures the activity of hydrogen ions in a system.

The initial and intrinsic  $E_h$  value in any growth medium dictates development and progression of microbial growth. Similarly, microbial growth influences the reduction-oxidation state of a medium by means of the metabolic end-products, changes in pH and oxygen consumption (Fulde and Fabian 1955). This situation is of significant relevance in cucumber fermentation, where production of acid reduces the environment and prevents growth of spoilage and/or undesired microorganisms. In sauerkraut fermentation, unique redox potential patterns have been detected depending upon the microbial background.

During the switch from the initial domination of *Leuconostoc mesenteroides* to *L. plantarum* and the resulting alterations in organic acid and sugar concentrations, corresponding changes in  $E_h$  were observed (van Dijk and others 2000).

The use of a benchtop fermentor system allows for a high level of control during experimental fermentations, and allows for the regulated inclusion of gases. The use of both oxygen and hydrogen gases have been previously implemented to manipulate redox potential, and therefore influence the physiology of a fermentation. In prior research, the growth and acid production of both *L. plantarum* and *Leuconostoc mesenteroides* was delayed when hydrogen gas was used to create a reduced culture medium (Bourel and others 2003; Ouvry and others 2002). Growth and rate of acid production by yogurt probiotic cultures were also decreased in the presence of the reducing agent cysteine (Dave and Shah 1998). Alteration of a medium's redox potential has also been conducted using the reducing power of certain microorganisms, including *Enterobacter cloacae* (formally *Aerobacter cloacae*) (Fulde and Fabian 1955).

Since the practice of tank gas purging is currently being applied in the cucumber fermentation industry, it is necessary to further understand the effect that the purging has on the rate of microbial growth and acid production, as well as redox potential. An optimal  $E_h$  for growth of *L. plantarum* in cucumber fermentation is still unknown. The objective of this research was to evaluate cucumber fermentations where redox potential was manipulated through gas purging. It was investigated whether or not the growth of LAB could be influenced and if the onset of fermentation could occur more rapidly.

### **4.3. Materials and Methods**

#### **4.3.1. Bacterium and Medium**

Pure cultures of *Lactobacillus plantarum* LA0219 were obtained from the culture collection of the U. S. Department of Agriculture-Agricultural Research Service, Food Science Research Unit, located in Raleigh, North Carolina. *L. plantarum* LA0219 was incubated under aerobic conditions at 30 °C for 48 h using deMan, Rogosa, and Sharpe agar (MRS) (Becton, Dickinson and Co., Sparks, Md., U.S.A.). *L. plantarum* LA0219 has been previously isolated from fermented cucumbers and demonstrates tolerance to high salt and high acid conditions.

#### **4.3.2. Cucumber Fermentations**

Size 3B cucumbers (39 to 51 mm diameter) were obtained from a local processor. Cucumbers were rinsed, inspected visually, and sorted after being received, and then diced into 10 mm cubes by a Hobart FP150 Food Processor (Hobart Corporation, Troy, Ohio, U.S.A.).

Cucumbers and brine were packed into glass vessels of a BioFlo110 Modular Benchtop Fermentor (New Brunswick Scientific Co., Inc., Edison, N.J., U.S.A.) in a 50:50 (w/v) ratio. Two independent trials were conducted for each run. At equilibration, cucumber brine contained 0.05% (8.33 mM) acetic acid, 0.44% (40 mM) calcium chloride and 6% (1.03 M) sodium chloride (Morton Canning and Pickling Salt; Morton Salt, Chicago, Ill., U.S.A.). Vessels were inoculated with *L. plantarum* LA0219 or remained non-inoculated, allowing

natural fermentation to proceed. A constant temperature of 30 °C was maintained for each fermentation using a re-circulating chiller (NESLAB Merlin M-75, Thermo Electron Co., Newington, N.H., U.S.A.).

Nitrogen, oxygen, and hydrogen gases were sparged into the vessels, or flushed onto the headspace at a flow rate of 2.5 to 5 ml/min controlled by a Matheson PG-1000 (U001) flowmeter (Matheson Instruments, Montgomeryville, Pa., U.S.A.). Nitrogen was continuously sparged into the system. Oxygen and hydrogen were sparged into the system for 1 to 2 hours, allowing for the initiation of a change in redox potential. Gas sparging by oxygen and hydrogen was followed by a nitrogen headspace which was maintained for the duration of the fermentation.

#### **4.3.3. Measurement of Redox Potential and pH**

Both fermentation vessels were sealed off from the outside atmosphere. ORP (Oxidation-Reduction Potential) (Pt4805-DPAS-SC-K8S/200, Mettler-Toledo, Bedford, Mass., U.S.A.), pH (405-DPAS-SC-K8S/225, Mettler-Toledo), and dissolved oxygen (dO<sub>2</sub>) (Inpro 6830/220, Mettler-Toledo) electrodes were calibrated, rinsed with 70% ethanol and secured into fittings on the headplate of each fermentor vessel. The redox potential, pH, dO<sub>2</sub>, and temperature were continuously recorded using *BioCommand Plus* BioProcessing software (New Brunswick Scientific Co., Inc.).

The following equation was used to convert the ORP electrodes measurements to a standardized redox potential,  $E_h$  (van Dijk and others 2000).

$$E_h = E_{ref} + (RT / nF) \ln[H^+]$$

Where  $R$  is the gas constant ( $J K^{-1}$ ),  $T$  is the absolute temperature (K),  $n$  is the number of electrons involved ( $n = 1$ , for protons)  $F$  is the Faraday constant ( $C mol^{-1}$ ).

The simplified equation allows for normalization of the redox reading according to pH change, at 30 °C:

$$E_h = E_{ref} - 0.060126 \times pH$$

#### **4.3.4. Evaluation of Microbial Growth**

Fermentors were visually monitored for the development of turbidity and development of film yeast. After equilibration of the vessels, initial samples of cover brine solutions and cucumber cubes were collected using aseptic techniques for chemical and microbiological analyses. Samples were then collected every 24 h for 4 days to evaluate microbial growth.

Brine samples were plated on deMan Rogosa and Sharpe agar (MRS), Plate Count agar (PCA), and Yeast Morphology agar supplemented with 1 mM chlortetracycline and 1.5 mM chloramphenicol (YM/A). All culture media was from Becton, Dickinson and Co. (Sparks, Md., U.S.A.). Plating was conducted using a spiral plater (Autoplate 4000, Spiral Biotech Inc., Norwood, Mass., U.S.A.) and an automated colony counter (QCount, Spiral Biotech Inc.) was used to determine plate counts. MRS, PCA, and YM/A plates were incubated at 30 °C for 48 h. All media was incubated under aerobic conditions, except MRS plates that

possibly included a mixed microbial population. MRS plates were incubated anaerobically to prevent any yeast growth.

Microbial growth was also evaluated by determination of changes in sugars, organic acids, and ethanol during incubation of samples at 30 °C. Analyses were done by HPLC on a 30 cm HPX-87H column (Bio-Rad Laboratories, Hercules, Calif., U.S.A.) (McFeeters and Barish 2003). The column was heated to 65 °C and eluted with 0.03 N sulfuric acid at a flow rate of 1.0 ml/min. Samples were analyzed for propionate and butyrate using a UV6000 LP detector (Thermo Separation Products, Inc., San Jose, Calif., U.S.A.). A Waters model 410 refractive index detector (Waters, Milford, Mass., U.S.A.) connected in series was used to measure glucose, fructose, and ethanol.

For HPLC analysis, either brine or brine and cucumber cube samples (50:50 w/v ratio) were collected daily and frozen at -80 °C. Prior to analysis, samples were thawed, macerated as required, and centrifuged at 6,000 rpm using an Eppendorf 5810R Centrifuge (Eppendorf North America, Inc., Westbury, N.Y., U.S.A.).

#### **4.4. Results**

The trends in  $E_h$ , pH, microbial growth, carbohydrate utilization and organic acid production were compared for cucumber fermentation trials sparged with nitrogen, oxygen, and hydrogen or left untreated. The  $E_h$  values for a system sparged with hydrogen maintained a more reduced trend when compared to those with oxygen, nitrogen, or untreated. Minimal differences were observed in pH trends within the runs, despite the variations in  $E_h$  trends

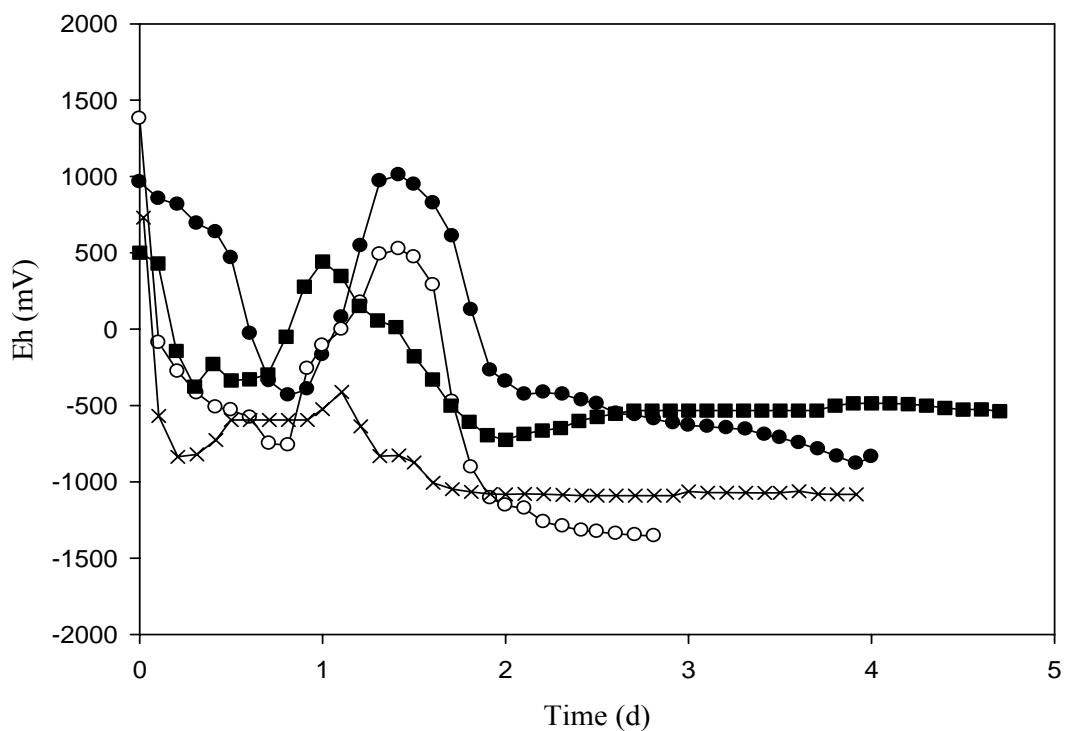
observed in the hydrogen sparged vessels. Similarly, microbial counts of lactic acid bacteria were comparable between treatments. However, vessels purged with oxygen showed higher yeast counts than others.

The general observed  $E_h$  trend for the vessels sparged with nitrogen, oxygen or maintained untreated showed a decrease of  $\sim 1500$  mV within the first day of fermentation followed by an increment of equivalent magnitude over 2 days, a second stage in which the measurements decreased and the establishment of a plateau around  $-500$  mV (Figure 4.1). Redox potential values for the vessels sparged with hydrogen, however, uphold a more reduced trend throughout the fermentation establishing a plateau at  $-975.4$  mV. As expected, the minimum  $E_h$  measurement of  $-975.4$  mV was established within a few hours of hydrogen gas sparging. At this time point (0.19 d), the  $E_h$  measured for each other treatment was considerably higher at  $+770.1$ ,  $-211.2$ , and  $-110.2$  mV for no treatment, nitrogen, and oxygen, respectively. As  $E_h$  increased following the first initial decline, the hydrogen sparged system reached a maximum of only  $-411.3$  mV (1.10 d), while maximums of  $+1023.8$  mV (no treatment, 1.38 d),  $+531.6$  mV (nitrogen, 1.40 d), and  $+462.9$  mV (oxygen, 1.01 d) were reached under other atmospheres.

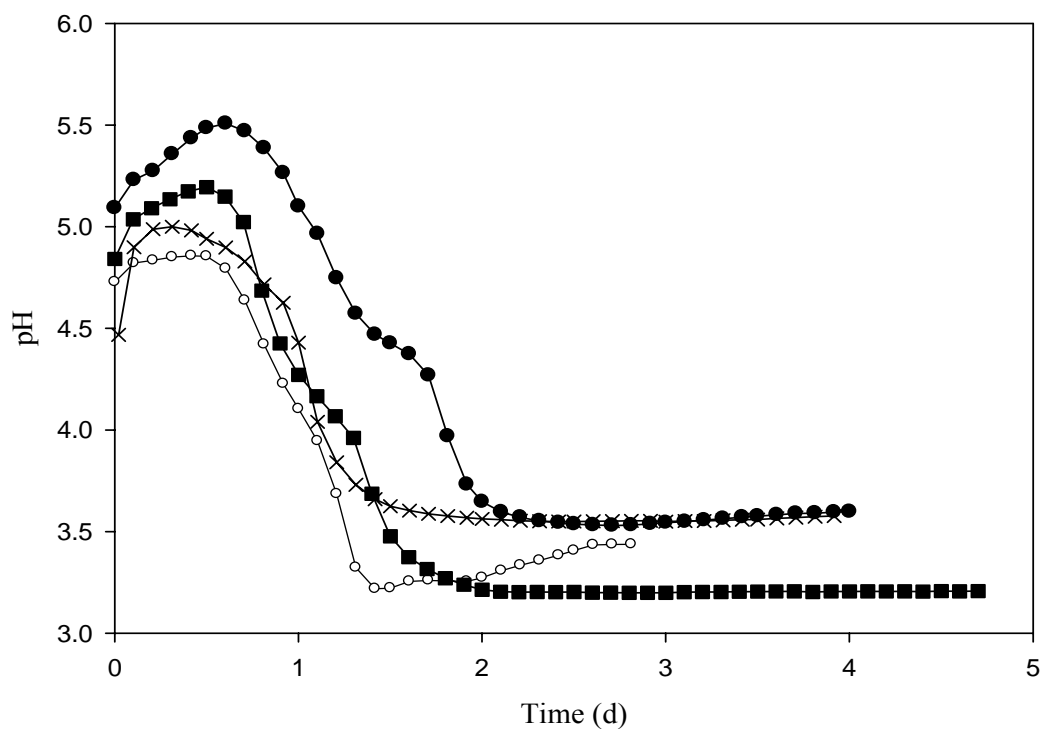
For each treatment, the pH values sharply decline within the first 24 hours and remain low and unchanged for the duration of the fermentation (Figure 4.2), measuring between 3.2 and 3.6 at the end of fermentation. The pH decreased at a faster rate during oxygen and nitrogen sparging, reaching final pH values of 3.2 and 3.4, respectively. Final pH measured at 3.6 under hydrogen sparging and when untreated.

Initial plate counts for lactic acid bacteria ranged from  $10^4$  to  $10^6$  CFU/ml (Figure 4.3) due to variations in the initial microbial load of the cucumber fruits and the fact that some vessels were inoculated. The vessels sparged with oxygen and hydrogen gas had the highest initial counts for lactic acid bacteria; however, growth occurred at a slower rate (0.170, and 0.191 generations/hr, respectively) between 0 and 2 d as compared to the other treatments. Counts of LAB reached  $10^8$  CFU/ml under all conditions. Initial plate counts for yeasts began between  $10^2$  and  $10^4$  CFU/ml (Figure 4.4). Trials with nitrogen, hydrogen, or no treatment had a maximum yeast count of  $10^6$  CFU/ml after 2 or 3 d. During sparging with oxygen, yeast colonies grew more rapidly (0.307 generations/hr), reaching levels of  $10^7$  CFU/ml after 1 d of fermentation.

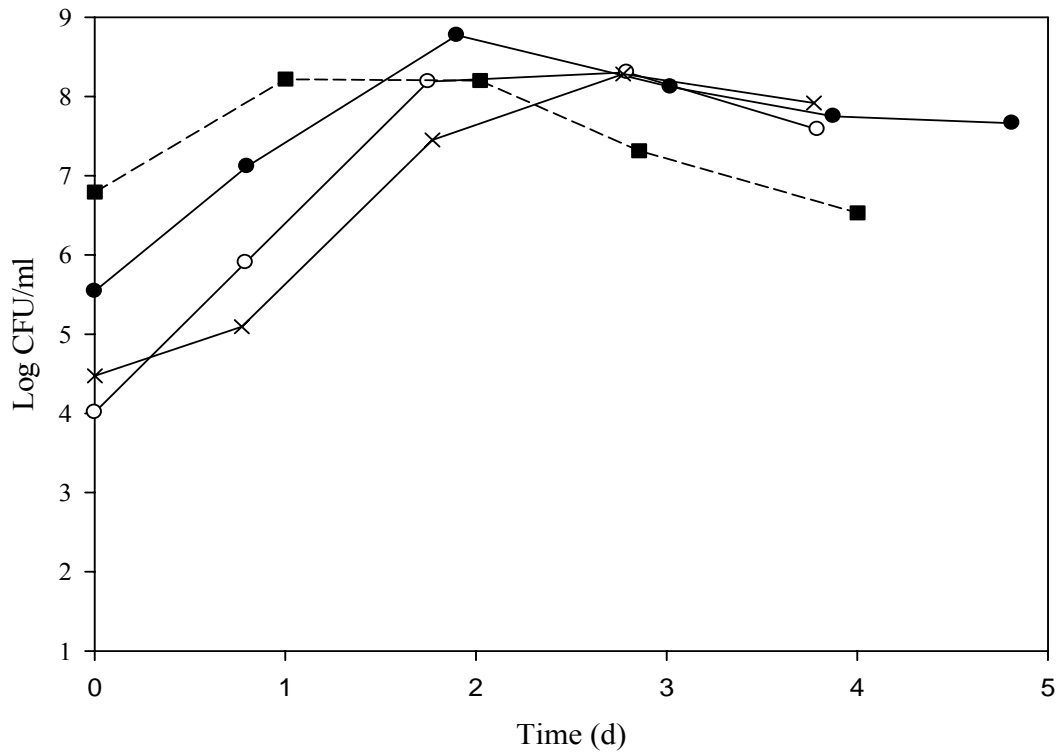
Available carbohydrates, in the form of glucose and fructose, were utilized within 2 d. Between 42 and 52 mM of lactic acid were produced in most cases, except for the hydrogen treatment, which generated 20 mM at a slower rate (0.170 generations/hr). Between 40 and 56 mM of ethanol were detected under each condition. No butyric or propionic acids were produced.



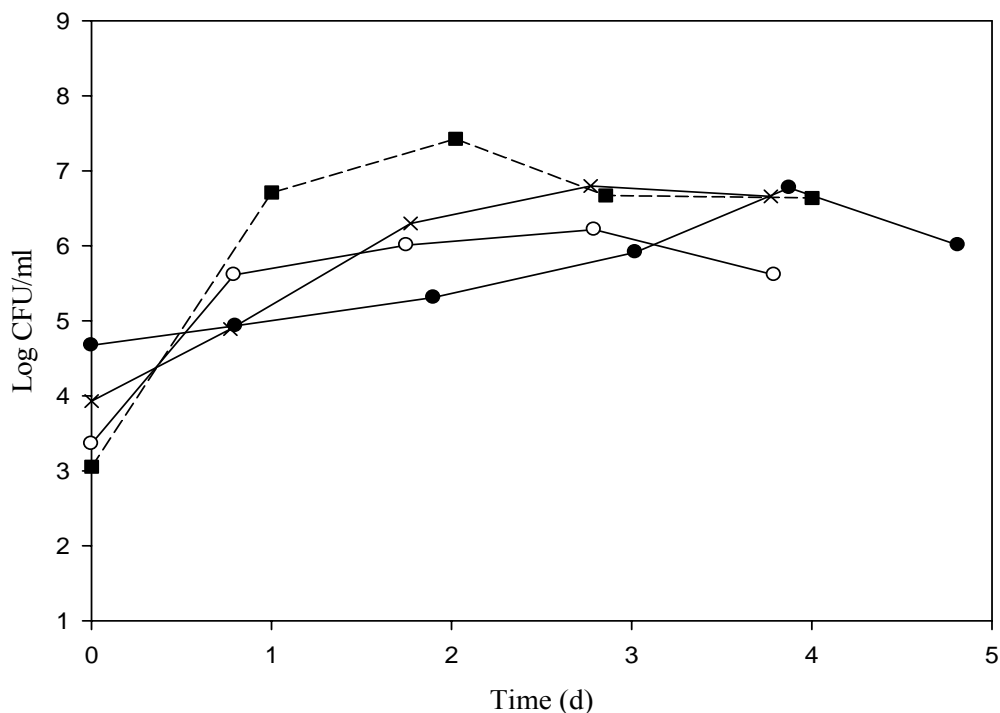
**Figure 4.1. Comparison of  $E_h$  trends during cucumber fermentations sparged with various gases.** Representative trends in  $E_h$  values with no treatment (●), nitrogen (○), oxygen (■) or hydrogen (x) introduced into the system.



**Figure 4.2. Comparison of pH trends during cucumber fermentations sparged with various gases.** Average trends in pH with no treatment (●), nitrogen (○), oxygen (■) or hydrogen (x) introduced into the system. Maximum standard deviations calculated were  $\pm 0.98$  pH units.



**Figure 4.3. Comparison of LAB growth during cucumber fermentations sparged with various gases.** Average microbial counts for LAB with no treatment (●), nitrogen (○), oxygen (■) or hydrogen (x) introduced into the system. Maximum standard deviations calculated were  $\pm 2.13$  log of CFU/ml.



**Figure 4.4. Comparison of yeast growth during cucumber fermentations sparged with various gases.** Average microbial counts for yeasts with no treatment (●), nitrogen (○), oxygen (■) or hydrogen (x) introduced into the system. Maximum standard deviations calculated were  $\pm 1.23$  log of CFU/ml.

#### 4.5. Discussion

The redox potential generated by the transfer of electrons between compounds in a system is capable of impacting the microbial activity within that environment. The microbial profile can also greatly affect the surrounding redox potential. The system is complex and minimal research has been conducted to further explore the relationship between microorganisms and redox potential, particularly in fermentation applications.

Although lactic acid bacteria, including *Lactobacillus plantarum*, are the predominate microorganisms in cucumber fermentations, there is a high level of variability in cucumber samples. The cucumber microflora changes as it is dependent upon factors such as the origin of the fruits, weather conditions, and storage climate. The initial microbial load naturally varies between trials, and also accounts for the possibility of yeast growth.

Redox potential measurements for fermentations with no treatment, nitrogen and oxygen sparging followed a comparable trend line, despite a high degree of variability due to the intrinsic differences in cucumber samples. However, initial hydrogen sparging worked to maintain a lower, more reduced  $E_h$  trend for the duration of the fermentation. Similar and less variable pH trends were observed, even though changes in  $E_h$  measurements were still detected, which suggest that redox potential may be a more discriminative approach as compared to pH for the examination of cucumber fermentations in the field.

The maintenance of a reduced environment did not favor LAB growth, as a high final pH (3.6), lowest growth rate (0.170 generations/hr) and lowest lactic acid production (2.62 mM lactic acid/Log CFU/ml) were observed after hydrogen sparging. In this harsh environment, bacterial cells appear to be spending more energy in the production of biomass, reaching a maximum of  $7.73 \pm 0.77$  Log CFU/ml, than in the production of lactic acid.

The optimum redox potential for the growth of lactic acid bacteria remains unknown. The growth rate (0.339 generations/hr) and efficiency of lactic acid production (6.69 mM lactic acid/Log CFU/ml) was the highest under nitrogen sparging; however, the  $E_h$  trend parallels that of oxygen sparged and untreated vessels. Nitrogen sparging appeared to be the best

overall treatment for cucumber fermentations, with the lowest ethanol production efficiency and highest efficiency in carbohydrate utilization, in addition to the fastest and highest acid production. Further research to determine the optimum redox potential using nitrogen sparging may prove beneficial for cucumber fermentations in the field.

#### **4.6. Conclusions**

Growth of LAB and lactic acid production were accelerated under nitrogen sparging, showing the most promise as an applicable tool in industrial fermentations.

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Boeriu C. 2000. Product and Redox Potential Analysis of Sauerkraut Fermentation. J Agric Food Chem 48(2):132-9.

**Chapter 5.**  
**Conclusions**

Fermented foods and beverages are popular worldwide. These products, including pickles, often rely on natural fermentation processes based on indigenous lactic acid bacteria (LAB). LAB utilize carbohydrates available to produce lactic acid, lower the pH of their surroundings and preserve the products, while creating a unique flavor profile. Cucumber fermentations are often spoiled by the growth of yeasts, which are not detectable by pH measurements. Evaluating the changes in redox potential ( $E_h$ ) during fermentation offers an alternative to traditional pH assessment. While pH is defined as the negative logarithm of the concentration of hydrogen ions in a solution, redox potential is defined as the ability of a system to be oxidized (gain electrons) or reduced (lose electrons). By definition, redox potential has the capability to offer a broader portrayal of the metabolic changes that occur over the course of a fermentation. Thus,  $E_h$  measurements could be used as a tool for the improved prediction of spoilage in cucumber fermentations.

In the case of pasteurized and inoculated jars of fermenting cucumbers, as well as non-pasteurized jars, unique  $E_h$  trends were discovered between fermentations by lactic acid bacteria and by fermentations that suffered yeast spoilage.

During gas purging in 2 L fermentor vessels, the overall  $E_h$  trend under hydrogen sparging remained more reduced than trends for treatments which received no treatment, or were sparged with nitrogen or oxygen. It was with the inclusion of nitrogen that the highest growth rate for lactic acid bacteria was found. In this treatment, the highest overall efficiency for lactic acid production was measured, as well as the lowest efficiency for production of ethanol between the gas treatments.

Although *Enterobacter aerogenes* produces hydrogen gas as a byproduct, it was not able to influence the  $E_h$  trend in the same way as hydrogen gas sparging. When in the presence of *Lactobacillus plantarum*, *E. aerogenes* was not able to control and maintain a reduced  $E_h$  environment. Hydrogen gas sparging, with natural LAB growth, was able to maintain the reduced  $E_h$ .

Despite carbohydrate exhaustion, the death phase of the LAB life cycle was not observed in all of the trials. For shorter fermentations, LAB adjusted to their acidic environments and remained in stationary phase.

The patterns of redox potential changes were different when different microorganisms dominated the fermentation, and they did not parallel changes in pH. The results suggest that  $E_h$  may be useful in detecting excessive yeast growth in cucumber fermentations.

Future research is necessary to be able to apply this technique in the industry. Evaluation of redox potential trends in large scale bulk tanks of fermented cucumbers needs to be conducted to verify this work in a real world setting. If using redox potential to predict spoilage in brined cucumbers proved advantageous, then this tool could be expanded to use with other fermented foods and beverages.