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Evaluation of an amperometric biosensor for the detection of Escherichia coli O157:H7

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EVALUATION OF AN AMPEROMETRIC BIOSENSOR FOR THE DETECTION OF *ESCHERICHIA COLI* O157:H7

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ABSTRACT

Escherichia coli O157:H7 contamination is a major hazard in the water supply, causing outbreaks of disease. Conventional methods of *E. coli* O157:H7 detection usually take 1-2 days and require hands-on preparation. There is a need to develop a rapid, inexpensive means of detecting the organism. The amperometric biosensor technology has achieved success in the area of metabolite detection. In this study, a bench scale amperometric biosensor was investigated to rapidly detect *Escherichia coli* O157:H7. The amperometric biosensor consisted of a power source, Clark electrode, picoammeter, and fabricated polyvinyl chloride (PVC) outer insert with nitrocellulose membrane and attached horseradish peroxidase labeled *E. coli* antibodies. The interaction of horseradish peroxidase and hydrogen peroxide produced dissolved oxygen, which is anticipated to be altered by the binding of the antigen to the antibody. After submerging the amperometric biosensor in the samples containing various concentrations of heat sterilized *E. coli* O157:H7 cells, as little as 10 cells/ml of *E. coli* O157:H7 were detected. The time for detection for the final system was approximately 20 minutes. There was a need to use a custom conjugated antibody to control and increase the molar concentration of conjugated HRP. The minimum concentration of HRP needed for this system was 6 X 10^{-8}M HRP. The system showed optimal performance at pH values 6-8 and at temperatures 10-30°C and showed no response in acidic environments with pH values less than 5. The results indicated that change in dissolved oxygen response can be used to distinguish between 0 and 10-5000 cells/ml. Maximum increases in dissolved oxygen of 3.53mg/L ± 0.26mg/L when bacterial cells were present and increase in the order of 6.26 ± 0.64mg/L when no cells were present was observed. Despite satisfactory

performance as an indicator method, the amperometric biosensor failed to quantify the organism. Further optimization experiments of the amperometric biosensor may be necessary for quantification. The amperometric biosensor with the use of a sandwich assay evaluated in this study offered a reliable means of quantification of the organism. Overall, the amperometric biosensor technology offered an efficient means of detection because of its ease of use and inexpensive, portable instrumentation.

CHAPTER 1. INTRODUCTION

1.1 Overview

E. coli O157:H7 is a type of fecal coliform bacteria that is known to be present in the gastrointestinal tract of cattle, mainly dairy calves. Unlike other fecal coliform bacteria, *E. coli* O157:H7 acts as an easy indicator for fecal coliform contamination. *E. coli* O 157:H7 displays qualities of an easy indicator due to its ability to persist in a significantly larger range of environments than most other fecal coliform bacteria. Common sources of contamination of *E. coli* O157:H7 include contaminated surface and ground water sources due to urban and agricultural runoffs. Ultimately, contamination with this organism can result in lowered water quality and increased human fatality.

From a health and safety perspective, *Escherichia coli* O157:H7 has many unique characteristics that distinguish this strain of *E. coli* from others. The first distinguishing factor is that it is one of the few strains of *E. coli* that can cause renal damage, possibly resulting in death. The second distinguishing characteristic is that it is persistent in the environment. *E. coli* O157:H7 is known to be able to survive in very low temperature and at very low pH. From documented reports, the fate of *E. coli* O157:H7 in bovine feces revealed that the pathogen survived for 42 to 49 days at 37°C, for 49 to 56 days at 22°C, and 63 to 70 days at 5°C (Wang, et al, 1996). The third distinguishing factor is its very small infective dose**.** As little as 10 to 100 *E. coli* O157:H7 cells are sufficient to cause disease (H. Petridis, et al, 2002).

1.2 Impact on Louisiana

Many ground and surface water sources across Louisiana are experiencing lowered water quality due to organism contamination. One example of this problem is

the south shore area of Lake Pontchartrain in Orleans Parish. In 1985, a primary contact recreation advisory was issued that named Fecal Coliform (FC) bacteria as the causative pollutant. The primary suspected source of the bacterial pollution is pumped urban storm water runoff contaminated by sanitary sewer cross-flows that is discharged to the area (U.S Geological Survey, 2004). Five monitoring stations have been set up in order to study water quality. In this study, fecal coliform bacteria were used as indicators of polluted recreational water. The amount of coliform bacteria present was directly correlated with the extent of pollution. *E. coli* O157:H7 was measured in this study using conventional methods. The current conventional methods usually require hands-on preparation and 24 to 48 h of incubation time before the pathogen can be identified and quantified (Jay, 2000).

There are many rapid detection methods which are being explored to detect *Escherichia coli* O157:H7. Some of these methods include immunological detection, PCR (polymerase chain reaction) based methods, fluorescence, and microscopy. These methods offer many advantages in the area of detecting *Escherichia coli* O157:H7. One drawback to rapid detection methods is that they usually require many steps including a lengthy enrichment process. This enrichment process may include separation and extraction techniques, and sample growth in media selective for *E. coli* O157:H7. However, biosensors are usually known to provide real-time measurements and allow rapid analysis time. Furthermore, biosensors are not usually known to be associated with lengthy enrichment processes. Though biosensors offer many advantages to current rapid detection methods, there is still a lot of room for growth in this field. "For biosensors, commercial developments have been slow as a result of the intense competition from

other methods, and the intrinsic difficulties in rendering the technology the technology sensitive and reliable enough" (Deisingh and Thompson 2002).

There are many types of biosensors currently being explored for *E. coli* O157:H7. Among these are fiber optic biosensors and surface plasmon resonance (SPR) biosensors. These biosensors, like other rapid detection methods, have many advantages and disadvantages, which parallel those of general biosensors. Some advantages include real-time detection capabilities and total detection (preparation and detection) of one hour or less and low organism detection limits. Some disadvantages to the systems include complex and expensive instrumentation which may require some degree of specialization for use. There is much room to explore other types of biosensors. For instance, there has been much success with amperometric biosensors for the detection of metabolites, most commonly glucose. Generally, glucose amperometric biosensors are composed of a base transducer which is normally a hydrogen peroxide or oxygen sensor, an inner membrane selective for hydrogen peroxide or oxygen, and an outer immunological membrane. Amperometric glucose biosensors are commercially available and a very effective means of glucose measurement. Amperometric biosensors to detect *E. coli* O157:H7 may offer a fast, reliable, and cost efficient way to quantify the organism. The amperometric biosensor technology is known for its ease of use, sensitivity, and quick response time. This will allow not only researchers, but station monitors to obtain quick and reliable results, and in effect produce quicker solutions to the water contamination problems in Louisiana and other areas. In this study, the use of an amperometric biosensor to detect *Escherichia coli* O157:H7 will be explored. Further objectives for this study are outlined below.

1.3 Objectives

The purpose of this research was to evaluate the effectiveness of an amperometric biosensor with a single labeled antibody for the detection of *Escherichia coli* O157:H7. A series of bench scale laboratory experiments were conducted on the fabricated amperometric biosensor system. Specific goals of this research were as follows:

1. To construct an amperometric biosensor system to detect *Escherichia coli* O157:H7 for bench scale laboratory testing.
2. To evaluate the amperometric biosensor system performance to detect *Escherichia coli* O157:H7 as a function of:
    a. Solution pH and temperature- Correlate the signal generated with the effect of changing pH and temperature. The pH and temperature range commonly found in bodies of water in southern Louisiana will be compared with the range of use for the amperometric biosensor.
    b. Enzyme Concentration-Correlate signal generated within a range of enzyme concentrations. Evaluate change in dissolved oxygen that can be achieved with the use of a hydrogen peroxide.
    c. Bacteria Concentration- Correlate signal generated with varying concentrations of *E* .coli O157:H7. Concentrations of *Escherichia coli* O157:H7 will be detected using amperometric biosensor system by evaluating current response.

3. To evaluate overall effectiveness of amperometric biosensor system to detect *Escherichia coli* O157:H7. The response time and sample preparation will be evaluated.

4. To evaluate system limitations for *E. coli* O157:H7 detection using the amperometric biosensor setup. These limitations may include range of detection and detection capabilities of the system.

1.4 Scope

The amperometric biosensor to detect *E. coli* O157:H7 may have many possible uses in the fields of environmental sampling, food pathogen detection, and biomedical detection of the organism. This amperometric biosensor system explores the feasibility of using an amperometric biosensor for the detection of *Escherichia coli* O157:H7. Ideally, such an amperometric biosensor could be used at a monitoring station like those found at Lake Pontchartrain in southern Louisiana. Commonly, monitoring stations allow analysis for variables such as temperature, chemical oxygen demand (COD), biomass oxygen demand (BOD), and pH. Successful testing of the proposed amperometric biosensors allowed identification of source contamination which seems to be a major concern for many lakes.

Chapter 2. Review of Literature/Background

2.1 Classification of *E.coli* O157:H7

There are six recognized classes of diarrhengenic *E. coli*: enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), entereroaggregative (EaggEC), enteropathogenic (EPEC), and diffusely adherent (DAEC). *E. coli* O157:H7 is categorized as enterohemorrhagic (EHEC). EHEC strains are defined by their virulence factors and symptoms they produce (Neill et al, 1994). Hemorrhagic Colitis (HC), also known as bloody diarrhea, is the defining symptom of EHEC. Though *E. coli* O157:H7 produce a high rate of bloody stool, this is not the case for all EHEC strains. A common factor amoung EHEC strains are the toxins produced. For example, all EHEC produce Shiga toxin 1 (Stx 1) and /or Shiga toxin 2 (Stx 2), also referred to as verotoxin 1 (VT1) and verotoxin 2 (VT2), which was acquired from a bacteriophage, possibly directly or indirectly from *Shigella*. The toxin is 70,000 dalton protein composed of a single A subunit (32kDa) and five B subunits (7.7kDa). Tissue specificity binding is provided by the B subunit, while the A subunit blocks protein synthesis. Tissue specificity is achieved by binding to globotriaosylceramide (Gb_3) receptors on the surface of eukaryotic cells. The primary target of the toxin is endothelial cells which are high in Gb_3. Because toxin alone is insufficient to categorize E coli pathogenic, EHEC requires the presence of other virulence markers. One example of another virulence marker for *E. coli* is the *eae* chromosomal gene associated with attachment (Buchanan and Doyle, 1997).

2.2 Disease Characteristics

The initial symptoms of hemorrhagic colitis can be seen 1-2 days after consuming contaminated food. Symptoms start with mild, non-bloody diarrhea that may include cramp-like abdomen pain and short-lived fever. After which in the next 24-48 hour period , a 4-10 day spell of overtly bloody diarrhea would be experienced followed by severe abdomen pain and moderate dehydration (Buchanan and Doyle, 1997). Figure 2.1 outlines the overall symptoms and time course associated with *E. coli* O157:H7 infections.

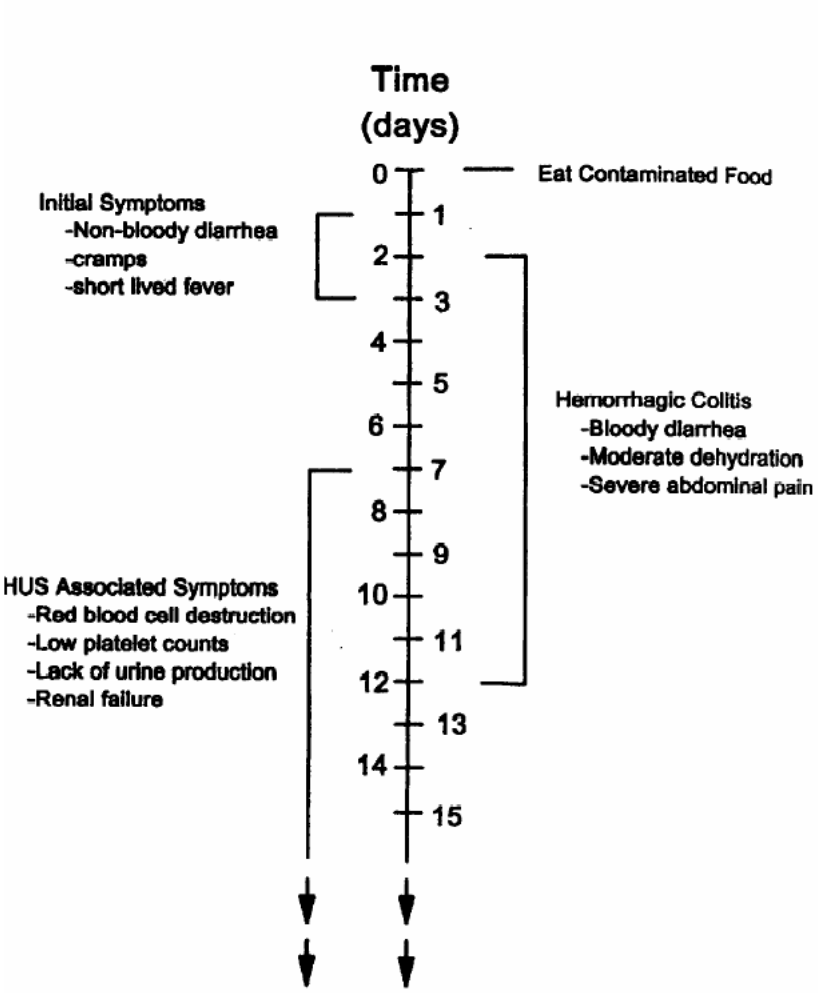

Figure 2.1: Symptoms and Time Course of *Escherichia coli* O157:H7 infection (hemorrhagic colitis) and its primary complications (hemolytic uremic syndrome, HUS
Source: Buchanan and Doyle (1997)

Some life threatening complications may occur in HC patients. The most common is hemolytic uremic syndrome (HUS). Some symptoms associated with HUS include: pallor, intravascular destruction of red blood cells, depressed platelet counts, lack of urine formation, swelling, and acute renal failure. Other symptoms associated could include seizures, coma, stroke, colonic perforation, pancreatitis, and hypertension. Approximately half of HUS patients require dialysis and the mortality rate is 3-5 percent (Buchanan and Doyle, 1197). Another life threatening complication that can occur is called thrombotic thrombocytopenic purpura. This condition generally causes less renal damage than HUS and significant neurological involvement. Thrombotic thromboctopenic pupora can generally result in central nervous system deterioration, seizures, and strokes, and is restricted primarily to adults (Boyce et al., 1995).

2.3 Sources of *E. coli* O157:H7

2.3.1 Cattle

Cattle have been identified as one of the main reservoirs and sources of *E. coli* O157:H7. This was concluded after further investigation of *E. coli* O157:H7 infections associated with undercooked beef and raw milk. After further investigation, some generalizations were obtained related to cattle based infections (Buchanan and Doyle, 1997). *E. coli* is carried more frequently in younger cattle than adult cattle (Zhao et al., 1995). Incidence of *E. coli* O157:H7 varies widely because of the use of different detection procedures (Buchanan and Doyle, 1997). The range of *E. coli* O157:H7 in cattle manure is in the range anywhere from 10^2 to 10^5 CFU/g (Zhao at al., 1995). In a single herd, more than one strain of *E. coli* O157 can be identified within one animal or among different animals (Faith et al., 1996; Meng et al., 1995).

Experiments have been conducted in which calves were infected with *E. coli* O157:H7. From these results it can be concluded that *E. coli* O157:H7 is not pathogenic to calves. It was also concluded that the number of *E. coli* O157:H7 shed from cattle feces decreased dramatically after the first 14 days post inoculation. This study found that *E. coli* O157:H7 was confined to the gastrointestinal tract. In some animals, fasting decreased the shedding of *E. coli* O157:H7 in feces. E.coli O157:H7 did not colonize mucosal surfaces and did not form attaching legions (Brown et al., 1997; Cray and Moon, 1995).

2.3.2 Deer and Sheep

Recently, deer have been named as another source of the pathogen O157:H7. It is thought that transmission of the pathogen could possibly be passed between sheep and cattle (Keene et al., 1997: Rice et al., 1995).

Sheep have also been identified as a source of the pathogen *E. coli* O157:H7 (Kudva et al., 1996). After conducting a six month study, it was revealed that fecal shedding of the pathogen from sheep was both transient and seasonal. The sheep showed no sign of disease throughout the study and shedding of the E.coli O157:H7 administered showed signs of shedding for up to 92 days (Kudva et al., 1995).

2.3.3 Water

Recreational and Drinking water supplies have been reservoirs for *E. coli* O157:H7 allowing for the transmission of the pathogen and outbreaks of infection (Doyle et al., 1997). There have been many documented cases in which water supplies have resulted in outbreaks of infections. One contaminated municipal water supply reported in Carbool, Missouri resulted in the 243 cases of outbreaks which included four deaths

(Swerdlow et al., 1992). In Portland, Oregon, a contaminated lakeside park swimming area left 21 cases of *E. coli* O157:H7 infections (Keene et al., 1994).

2.3.4 Foodborne

Food, including fresh or undercooked ground beef, appears to be one of the primary sources of human infections (Doyle and Schoeni, 1984). Foods usually associated with the transmission of *E. coli* O157:H7 may be attributed to person-to-person (Griffin and Taux, 1991) or animal-to-person (Wilson et al, 1996) spread of E.coli O157:H7 and other enterohemorrhagic E.coli (Buchanan and Doyle 1997). Table 2.1 list foods or food handling practices suspected of being associated with *E. coli* O157:H7 outbreaks.

Table 2.1: Foods or Food handling practices implicated or suspected of being associated with *Escherichia coli* O157:H7 outbreaks
Source: Buchanan and Doyle (1997)

Undercooked ground beef
Raw milk
Unpasteurized apple juice/cider
Dry cured salami
Lettuce
Produce from manure-fertilized garden
Handling potatoes
Radish sprouts, alfalfa sprouts
Yogurt
Sandwiches
Water

2.4 Factors Affecting *E. coli* Survival and Growth

2.4.1 Temperature

Temperature is one of the most important factors affecting microbial growth and survival. Microorganisms can grow in temperatures varying from below freezing to over 100° C. Microorganisms can be classified as mesophiles, psychrophiles, themophiles, or extreme thermophiles based on their ideal temperature needed for growth. The Arrhenius equation is utilized to related microbial growth to temperature (Bitton, 1999).

E. coli, unlike other *Enterobacteriaceae*, are able to grow and produce gas in EC broth at 44.5°C (Buchanan and Doyle, 1997). *E. coli* O157:H7 isolates do not usually grow above 44°C (Doyle and Schoeni, 1984). The exact upper temperature for *E. coli* O157:H7 is dependent upon the type of medium it grows on (Buchanan and Doyle, 1997). The minimum temperature for growth is between 8-10°C (Buchanan and Bagi, 1994; Rajkowski and Marmer, 1995).

2.4.2 pH

In general, the optimum pH for bacteria growth is around neutral pH (pH 7). Bacterial growth usually causes a decrease in medium pH due to the releasing of acidic metabolites, though some bacterial growth increases the pH of the medium. The pH level affects the activity of the microbial enzymes by playing a role in transport of nutrients and toxic chemicals into the cell (Bitton 1999).

For *E. coli* O157:H7, growth rates are similar at pH levels between 5.5 and 7.5. This growth does decline at lower pH values, with the minimum pH needed for growth being between 4-4.5 (Buchanan and Klawitter, 1992; Buchanan and Bagi, 1994). The type of acid and acid concentration can affect the pH values needed for growth

(Buchanan and Doyle, 1997). For example, Abdoul-Raouf (1993) reported that inhibitory activity of organic acids on *E. coli* growth was aceteic>lactic>citric. *E. coli* O157:H7 is particularly well known for being able to survive at relatively low pH values. This is evident by the fact that *E. coli* O157:H7 is able to survive in foods that maintain low pH values such as fermented sausage, apple cider, and apple juice, and cheddar cheese (Zhao and Doyle, 1994; Clavero and Beuchat, 1996; Reitsma and Henning, 1996). Acid tolerance of *E. coli* O157:H7 is dependent upon growth phase (see microbial growth curve; Figure 2.2). In stationary phase, *E. coli* O157:H7 isolates are more tolerant than in exponential phase due to the expressions of genes regulated by the *rpoS* sigma factor operon (Buchanan and Doyle 1997; Cheville et al., 1996; Rowbury et al., 1996; Small et al., 1994). The period of acid tolerance can persist for 28 days or greater during refrigerated temperatures. The induction of acid tolerance can be linked to ability of *E. coli* O157:H7 to resist heating, radiation, and antimicrobials (Rowbury, 1995). Rowbury et al (1996) also found *E. coli* O157:H7 to have an alkaline response.

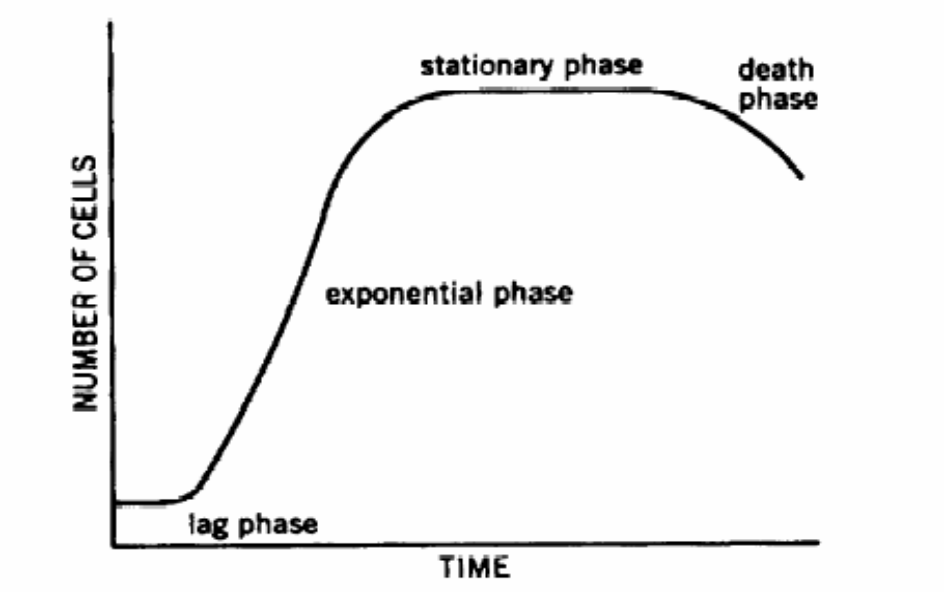

Figure 2.2: Microbial Growth Curve
Source: Bitton (1999)

2.5 Infectious Dose

Anyone can be infected by *E. coli* O157:H7, but the very young and elderly are the most vulnerable. The elderly and very young may be most affected because of decreased immunity and sanitation practices. The infectious dose of *E. coli* O157:H7 is from 50-100 organisms (Singleton 1995).

2.6 Methods of Detection

2.6.1 Conventional Methods

Commonly, indicator organisms like *E. coli* O157:H7 can be detected using a total coliform number. "Total coliform group includes all the aerobic and facultative anaerobic, gram-negative, non-spore-forming bacteria that ferment lactose with gas production within 48h at 35°C (Bitton, 1999)".

One method of detecting the total coliform group is the use of Most Probable Number (MPN). MPN is a statistical estimate of the concentration of an organism based on the application of the Poisson's distribution of extreme values to the analysis of the number of positive and negative results. These positive and negative results are obtained when testing various portions of equal volume and in geometric series. The MPN can be determined using Poisson distribution directly, MPN tables, or the Thomas equation (McGraw- Hill 1991).

MPN is often found via the multiple-tube fermentation technique. "The multiple tube fermentation technique is based on the principle of dilution to extinction" (McGraw-Hill, 1991). With the multiple tube fermentation technique, once a series of dilutions are made, a given amount, commonly one milliliter is transferred into five fermentation tubes. The fermentation tubes contain liquid media suitable to grow total specific

bacteria. Often fermentation tubes contain lactose and an inverted gas collection tube. The fermentation tubes usually take an inoculation period of 24 hours at 35°C. However, for fecal coliform bacteria, solid medium is often used especially where there is a use for an approximation of the fecal coliform bacteria count. The fecal coliform bacteria is often incubated at 35°C for 3 hours then incubated in a water bath at 44°C for 21 hours. Figure 2.3 shows the multiple-tube fermentation technique with the use of liquid and solid medium (McGraw-Hill, 1991).

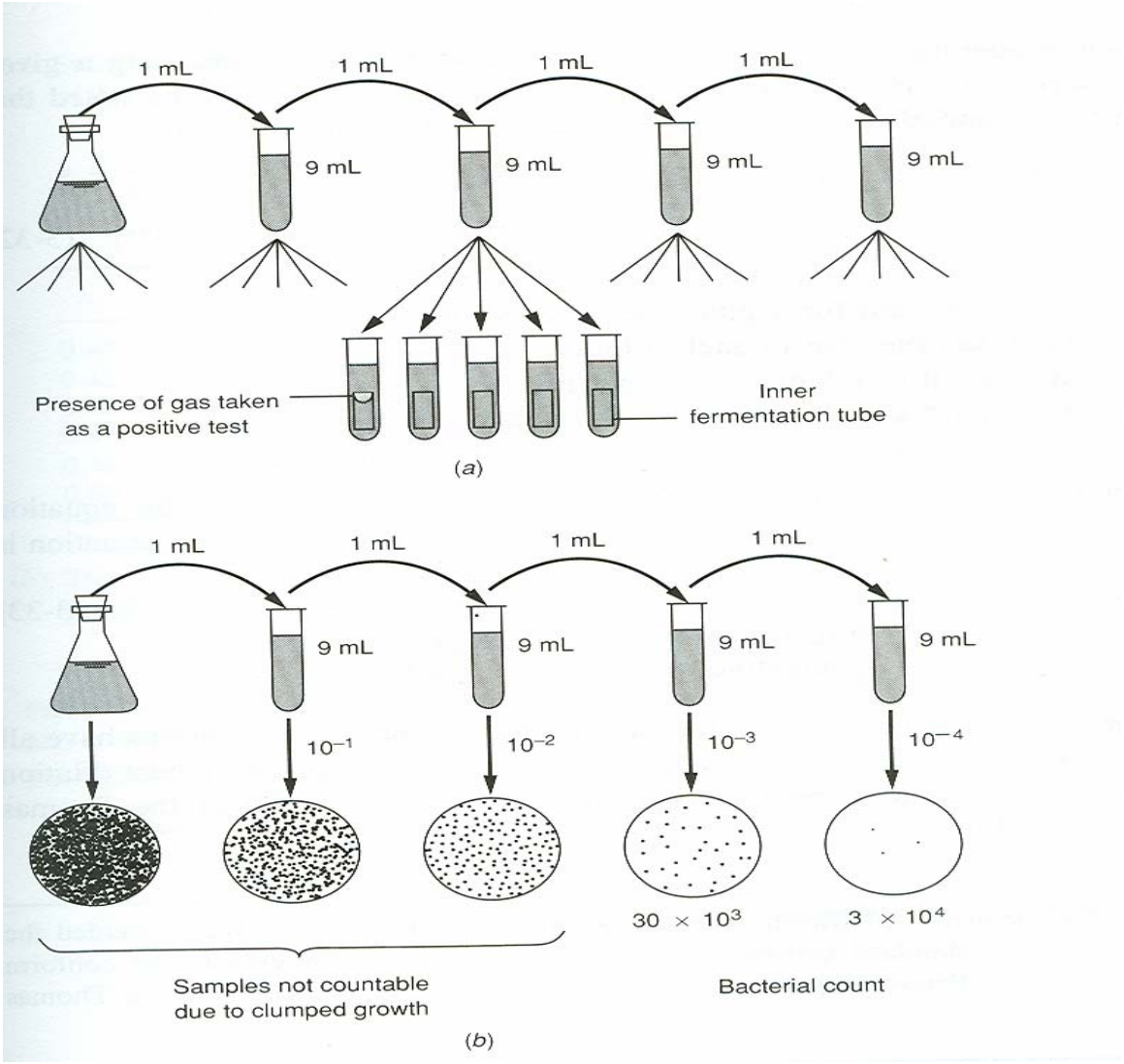

Figure 2.3: Illustration of methods used to obtain bacterial counts: (a) use of a liquid medium and (b) use of a solid medium
Source: McGraw-Hill, 1991 and Streeter and Phelps, 1925

Another standard method used to detect total fecal colifom bacteria is the membrane-filter technique. This method involves passing a water sample with a known volume through a filter with a pore size smaller than the bacteria in order to trap the bacteria in the filter. The bacteria are added to an agar with nutrients needed for growth. After incubation, the bacteria are counted on the solid medium much like the multiple tube fermentation dilution technique. The membrane filter technique gives a more direct count of the number of coliforms and slightly faster then the MPN technique (McGraw-Hill, 1991).

2.6.2 Rapid Detection Methods

2.6.2.1 Immunological Detection

One alternative approach to conventional methods is through the use of enzymatic assays. Commonly, *E.coli* enzymatic assays are based on the hydrolysis of fluorogenic substrates, namely 4-methylumbelliferone glucuronide (MUG) by β-glucuronidase, an enzyme found in *E. coli*. Using long-wave ultraviolet lamp, the fluorescent end product can be detected (Berg and Fiksal, 1988; Trepeta and Edberg, 1984). *E. coli* has been detected in both water and food samples utilizing this method by relating the fluorogenic compound to the most probable number (Feng and Hartman 1982; Robinson 1984). In this assay, the samples were incubated in lauryl-tryptose broth with 100 mg/L MUG for 24 hours at 35 °C and passed through membrane filters. The samples were observed for fluorescent illumination under a UV lamp. Within this 24 hour period, as small as one viable *E. coli* cell could be detected (Bitton, 1999; Feng and Hartman 1982; Robinson 1984). Hernandez et al (1990) utilized a similar assay and fluroscent method and observed a 87.3% confirmation rate.

The Autoanalysis Colilert (AC) test is used commercially to test both the total coliform count and *E. coli* cell counts in environmental samples (Covert et al., 1989; Edberg et al., 1988;1989;1990). The test consists of adding enzyme substrates 0-nitrophenyl- β-D-glucuronide (ONPG) and MUG, specific for detecting total coliform and *E. coli* cells respectively. Like the membrane filter enzyme assay technique, the process takes 24 hours. The MUG substrate and *E. coli* positive samples fluoresce under a long-wave UV illumination. After testing fecal samples, both animal and human, the test showed that 95% of *E. coli* isolates were positive after 24-hours (Rice et al., 1990). This test had a similar selectivity as the multiple tube fermentation method and the EC-MUG test (Covert et al., 1992; McCarty et al., 1992). The AC test did show a great deal of success; however, it also had many problems. One problem with the AC test was that not all *E. coli* strains, especially those found in human fecal samples were fluorogenic (Chang et al., 1989). Another problem with the AC test is that a certain percentage of *E. coli* producing virulence factors, for example enterotoxigenic and enterohemorrhagic *E. coli*, were not recovered on AC medium (Martins et al., 1992). In addition, some microalgae and macrophytes can produce β -galactosidase and β-glucuronidase,which in high concentrations could allow for false positive results (Davies et al., 1994).

ColiPAD^{TM} is also used to detect total coliform numbers and *E. coli* cells. This test is based on the hydrolysis of chlorophenol red-β-D-galactopyranoisde (CPRG) and MUG for the detection of total coliform and *E. coli* cells respectively. This detection method showed good results overall and achieved a good correlation, r^2 approximately 0.9, as compared to the standard tube fermentation method (Bitton et al., 1995).

Testing for *E. coli* using a proposed MUG based medium takes approximately 7.5 hours of incubation. This testing method for *E. coli* in water gave a specificity of 96.3 % (Sarhan and Foster, 1991). The use of chromogenic substrates is valuable for rapid and specific identification of *E. coli* on solid medium. The substrates indoxyl-β-D-glucuronide (IBDG) and 5-bromo-4-chloro-3-indolyl-β-D-glucuronode (X-Gluc) were found to be effective chromogenic substrates used to enhance detection of *E. coli* on solid medium (Gaudet et al., 1996; Watkins et al., 1988). Other enzyme substrates such as 4-methyl-umbelliferyl- β-D-galactopyranosidase for total coliform detection or indoxyl-β-D-glucuronide for detection of *E. coli* cells have been useful (Brenner et al., 1996). It was noted that the use of more sensitive enzyme-based methods to detect *E. coli* in less than 24 hours may compromise the specificity of the test (van Poucke and Nelis, 1997).

Gehring et al (1999) also tested a very useful enzymatic sensor to detect *E. coli* O157:H7. This sensor utilized magnetic beads and enzymatic sandwiching technique. This technique involved the use of the bacterial antigen between two antibodies, one which is specific for *E. coli* O157:H7 unlabeled and the other which is specific for *E. coli* O157:H7 and labeled with a phosphatase enzyme. After the substrate is added, the electroactive product was measured by square-wave voltammetry. The sensor was able to detect 4.7 X 10^3 cells ml^{-1} in approximately 80 minutes (Deisingh and Thompson, 2004).

The use of ELISA was also explored as a possible means an enzymatic essay to detect *E. coli* O157:H7. It was noted by Fratamico and Strobaugh (1998) that ELISA offered detection of 100 CFU ml^{-1} and had great sensitivity. But when compared with

techniques like PCR and direct immunofluorescent filter technique (DIFT), the drawback was the lengthy enrichment procedure which was at least 4 hours.

Monoclonal antibodies can be used to detect *E. coli* against outer membrane proteins or alkaline phosphatase (Joret et al., 1989). More research is needed to determine the feasibility of using monoclonal antibodies with *E. coli* samples in routine field samples. Some investigators question the specificity and affinity of the use of the monoclonal antibodies (Kfir et al., 1993)

Polymerase chain reaction is another type of molecular sensing technique**.** This method of sensing *E. coli* often uses specific genes found in the microorganism. The genes, for example, LacZ or lamb, are amplified by polmerase chain reaction and detected using a gene probe. Using polmerase chain reaction, *E. coli* can often be detected as low as 1-5 cells per 100ml of water (Atlas et al., 1989; Bej et al., 1990). Another type of polymerase chain technique for the detection of *E. coli* uses the uidA gene which codes for β̃-glucuronidase found in *E. coli* and Shigella. The uidA gene is detected using a probe and when combined with polymerase chain reaction can detect 1-2 cells but is unable to distinguish Shigella from *E. coli* (Martins et al., 1993; Bej et al., 1991a; Cleuziat and Robert-Baudouy, 1990).

In general, PCR has been somewhat successful in bacterial detection. Though it has offered a great deal of success, there are also many disadvantages. These disadvantages include the amplification of dead cells, complex data interpretation, and very intricate experimentation (Deisingh and Thompson, 2004). One example of this is noted by Uyttendaele et al (1999) in which a PCR assay targeting the 3'-end of the eae gene of the *E. coli* O157:H7 gene was able to detect 1pg DNA or 10^3 CFU PCR per

reaction. Sample preparation studies were investigated using various methods including centrifugation, buoyant density centrifugation (BDC), immunomagnetic separation (IMS), chelex extraction, and swabbing. It was found that IMS sample preparation did not produce false negatives, like the other methods, but only if they were below 108 $CFUg^{-1}$ (Deisingh and Thompson, 2004).

Multiplex and real-time PCR are variations of the standard PCR which seem to offer more sensitive detection. A multiplex PCR, which was able to detect viable cells and distinguish the serotype O157:H7, was used to detect *E. coli* O157:H7 in soil and water reported detection limits of 1 CFU ml^{-1} in drinking water and 2 CFU g^{-1} in soil (Campbell, 2001). In real-time PCR, with the use of a fluorogenic probe, the reaction is able to be characterized by the time amplification of the PCR product is detected (Livak, 2000). Reverse transcriptase PCR (RT-PCR) also offers a variation on PCR. The reverse transcriptase can detect 107 CFUs of the organism without the use of pre-enrichment, which reduces the time required for analysis (Yaron and Matthews, 2002).

The BAX® automated PCR system was developed by Du Point Qualicon (Wilmington, DE, USA). This system allows for the rapid detection of bacteria in raw ingredients, finished products, and environmental samples (Qualicon, 2001). The BAX® system combines the use of gel electrophoresis and PCR to determine of a specific target is present (Fritschel, 2001). The system contains a tablet which consists of all primers, DNA polymerase and deoxynucleotides for PCR, a positive control, and an intercalating dye. Instrumentation has been designed for the system in order to detect the fluorescent signal that is produced (Deisingh and Thompson, 2004). The instrument conducts the analysis to detect whether or not the bacteria is present. In using the BAX® system to

detect *E. coli* O157:H7, the BAX was found to be more sensitive than the conventional methods which had a detection rate of 39% compared with that of 96.5% with the BAX® system (Johnson et al 1998). One limitation of the this system was that it did not allow for quantification of the organism (Deisingh and Thompson, 2004).

2.6.2.2 Biosensors

Biosensor technology offers many advantages to organism detection and quantification including specificity, sensitivity, reliability, portability, real time analysis, and simplicity of operation (D'Souza, 2001) A biosensor is an analytical device that integrates biological sensing elements with electronic transducers (Turner, 1998). The main function of a biosensor is to convert biological events into an electronic signal (Cahn, 1993).

Fiber Optic biosenors are used in rapid detection of *E. coli* O157:H7**.** An evanescent-wave fiber optic biosensor was utilized to detect *E. coli* in 10g and 25g ground beef samples (Demarco and Lim, 2002). It was reported that the fiber optic biosensor was able to detect the 9.0×10^3 CFU g^{-1} in the 25g ground beef sample and 5.2×10^2 CFUg^{-1} in the 10g sample. It was reported that there were no false positives and that the results were obtained 25m after sample processing. Another fiber optic biosensor operating on an internal reflection format to detect genomic DNA from coliforms including *E. coli* reported that detection of fragments containing the lac Z sequence was obtained in approximately 20s by fluorescence measurements (Almadidy et al., 2002).

Surface Plasmon Renonasance biosensors are also available to detect *E. coli* O157:H7. BiaCore is an example of a surface plasmon resonance biosensor utilized to detect *E. coli* O157:H7. BiaCore was found to have a detection limit of 5×10^7 CFU ml^{-1}

(Fratamico et al 1997). This detection limit is not compatible with other methods of detection.

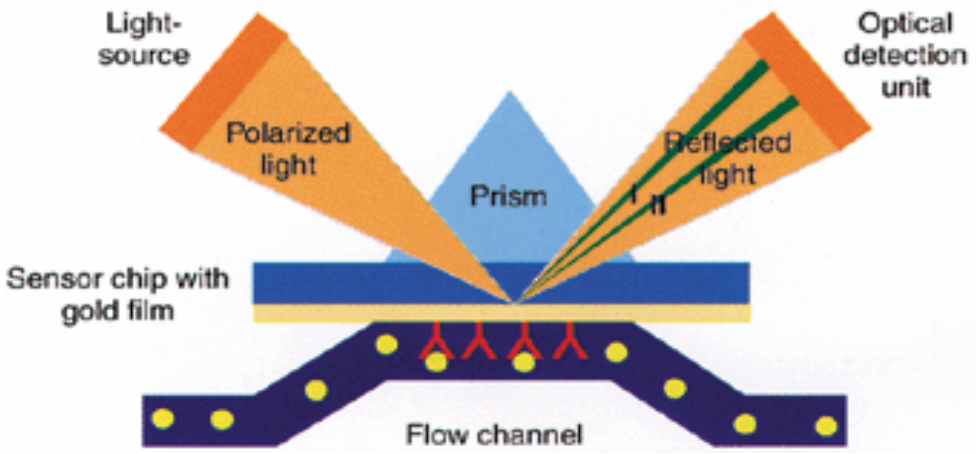

Figure 2.4: Schematic of the BIACORE surface plasmon resonance spectrometer
Source: Wang, 2004

Amperometric biosensors to detect *E. coli* O157:H7 may offer a fast, reliable, cost efficient way to quantify the organism. The amperometric biosensor technology is known for its ease of use, sensitivity, and quick response time. Amperometric biosensors are also known to be reliable, relatively cheap, and highly sensitive for environmental, clinical, and industrial purposes (Baronas et al, 2002). This will allow not only researchers, but station monitors to obtain quick and reliable results, and in effect, produce earlier solutions to the water contamination.

Amperometric biosensors work by creating a current once a potential is applied between two electrodes. The simplest form of the amperometric biosensor is used in junction with the Clark electrode. The Clark electrode is named after Leland Clark who first discovered the Clark type oxygen electrode. The Clark Electrode usually consists of a platinum cathode and a silver chloride reference electrode. Once a potential is applied (relative to the silver chloride electrode) to the platinum cathode as a result of oxygen

being reduced, a current is produced which is proportional to the oxygen concentration. In addition, the electrodes are usually saturated in a potassium chloride solution. The potassium chloride solution is usually separated from the bulk solution by an oxygen permeable membrane. The following reactions occur at the oxygen permeable membrane (Chaplin, 2003):

Ag anode $4Ag^0 + 4Cl^- \longrightarrow 4AgCl + 4e^-$

Pt cathode $O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$

The amperometric biosensor for this project utilizes a combination of the amperometric technology principle along with a substrate-enzyme complex. If a horseradish peroxidase (HRP) enzyme was conjugated with an antibody specific for *E. coli* O157:H7, the conjugated antibody would work as a biological receptor for *E. coli* O157:H7 bacteria. Once *E. coli* O157:H7 binds to the antibody conjugated with HRP, hydrogen peroxide could be added causing a product to be formed, namely oxygen (see reaction below). This oxygen formation would be able to be detected with a Clark electrode and could be correlated to the bacterial concentration.

$$\mathbf{H_2O_2} \xrightarrow{\text{HP}} \mathbf{O_2 + 2H}$$ HP = Horseradish peroxidase

There has been a successful attempt at an electrochemical immunoassay to detect E.coli O157:H7. This immunoassay consisted of a similar set-up to the amperometric biosensor with a few exceptions. The biosensor was based on a sandwich immunoassay using polyaniline conducting polymer. Two electrodes were placed at distance of 0.5mm apart, the optimum distance found between electrodes to optimize the signal generated.

An unlabeled antibody specific for *E. coli* O157:H7 was attached to a nitrocellulose membrane. The nitrocellulose membrane was an inner membrane on the biosensor. A known bacterial concentration was applied to an outer membrane which contained a second polyaniline labelled *E. coli* O157:H7 antibody. This formed an antibody-antigen complex. Through capillary action the antibody-antigen complex attached to the inner nitrocellulose membrane containing the unlabeled antibody, forming a sandwich. Once the sandwich was formed, the polyaniline forms a molecular wire between the electrodes which creates a signal. This signal was proportional to the amount of antigen, *E. coli* O157:H7. The disposable biosensor had many of the same advantages of other biosensors and was even able to detect as low as 7.8×10^1 colony forming units per millilitre (CFU/ml). Some problems with the biosensor was the inability to bind large amounts of the antigen (*E. coli* O157:H7), namely those which were greater than 10^4 CFU/ml. This is referred to as the over-crowding effect. The overcrowding effect caused a decreased signal at high concentrations which was most likely due to the interfering of unbound antigens with the electrons hopping between electrodes (Muhammd-Tahir and Alocilja, 2003).

Although this biosensor has much in common with the concept of the amperometric biosensor, there are some significant differences. Unlike the amperometric biosensor, the disposable biosensor utilizes a sandwich technique. The polyaniline on the labelled antibody is a conducting polymer which directly generates an electrical signal where the amperometric biosensor's signal is dependent upon an interaction of the labelled antibody and an added substrate.

2.7 Amperometric Biosensing System

The amperometric biosensor can interact with the system (water supply that may be contaminated) in two critical ways in water quality research. The amperometric biosensor can act as source identification in an open loop system or can be used in a closed loop system to control water quality. In a closed loop system an amperometric biosensor is used to signal a feedback response (actuator). This is depicted in the closed loop system below, Figure 2.5.

In an open loop system, the Amperometric biosensor could act as a first response in identification of fecal coliform contamination. This sensor could indicate that there may be some contamination coming from a water way (stream, canal, etc.) where an agricultural facility is found. This may initiate further investigation.

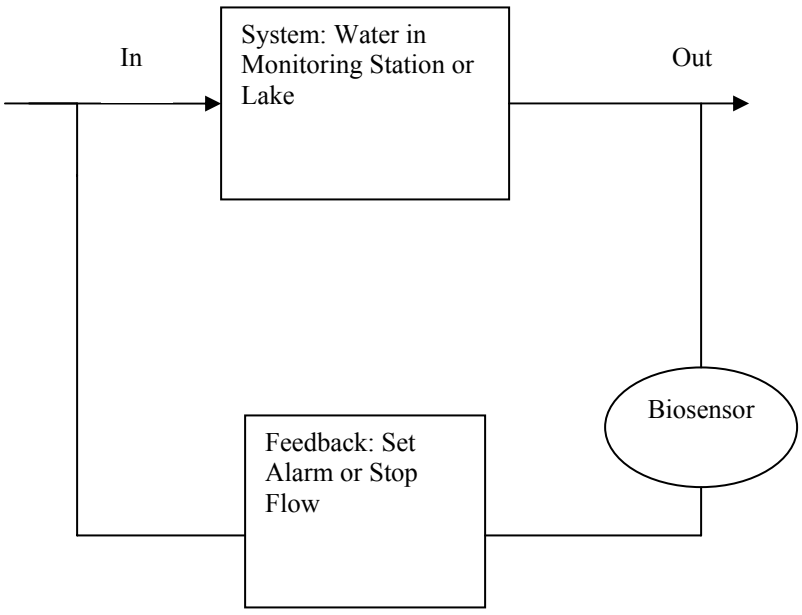

Figure 2.5: Closed Loop system using the Amperometric Biosensor

2.8 Single Antibody Amperometric Biosensors

There has been some success with the use of a single, peroxidase labelled antibody amperometric biosensor. Xu and Suleiman (1997) successfully created a single antibody amperometric biosensor to detect cortisol. It was found that with the binding of the antigen to a conjugated peroxidase antibody, the enzymatic activity of the peroxidase was decreased. This decrease in enzymatic activity was confirmed by luminescence testing. The biosensor was composed of a Clark Electrode with an outer membrane containing an immobilized peroxidase conjugated antibody. The biosensor offered several advantages including reusability, rapid response, and detection limit for cortisol of 1 X 10^{-7}M. The biosensor was also highly sensitive for the antigen of interest. No mechanistic proof was found on the steric hindrances causing decreased enzyme activity.

A similar amperometric biosensor was created by Xu and Suleiman (1998) which utilized conjugated HRP-antibodies. The amperometric biosensor was utilized to detect cocaine. This sensor showed rapid response, high selectivity, and simple analysis methodology. The calibration curve was linear from 1 X 10^{-7} to -1 X 10^{-5}M cocaine. Again, the amperometric biosensor experienced a decrease in enzyme activity with the binding of the antigen which was attributed to steric hindrance. This was confirmed by luminescence tests.

2.9 Microarrays, Molecular Beacons, and Integrated Systems

Microarrays and Molecular beacons are emerging technologies that may offer some advances in microbial detection. Microarrays allow rapid analysis, because thousands of specific DNA or RNA can be detected simultaneously on a glass slide 1-2 cm^2 (Aitman 2001). Some drawbacks to microarrays include instrumentation which is

expensive, very limited, and require specialized skill or training (Deisingh and Thompson, 2004).

Molecular beacons (MBs) may also offer significant progress in the area of detecting bacteria, namely *E. coli* O157:H7 (refer to Figure 2.6 for mechanism of action). McKillip and Drake (2000) used a beacon combined with PCR amplification to detect the pathogen in skimmed milk. By using the the combination of PCR and MB, they were able to obtain faster results than gel electrophoresis and allowed for real-time monitoring of PCR. The detection limit was 10^3 CFU ml^{-1}. Another use of molecular beacons with *E.coli* documented that it was possible to detect 10^2 CFU ml^{-1} in raw milk and apple juice without enrichment and with enrichment for 6 hours, detection limit improved to 1 CFU ml^{-1} (Fortin et al 2001).

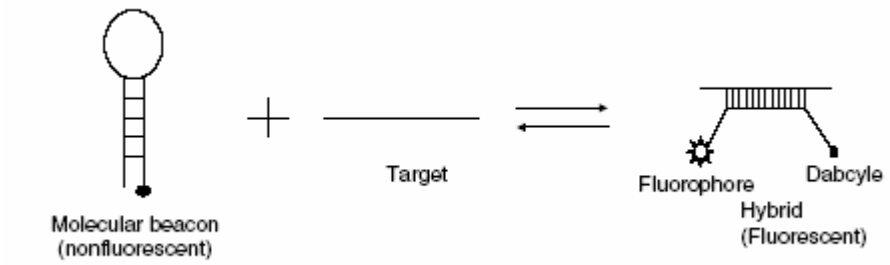

Figure 2.6: Principle of Detection of Hydrids with Molecular Beacons
Source: Deisingh and Thompson 2003

Intergrated systems, also known as lab-on-a-chip, may also be able to decrease analysis time and increase efficiency of detection (Deisingh and Thompson, 2004). One example of the use of an integrated system with the detection of pathogens is the integrated system which was described by researchers at the Lawerence Livermore

National Laboratory. The system uses the use of Advanced Nucleic Acid Analyzer (ANAA) to detect *Erwinia herbicola, Bacillus subtilis and B. anthracis*. The detection time was reported as short as 16 minutes and that 102-104 organisms ml^{-1} could be detected (Belgrader et al 1998).

There are many advantages and disadvantages to the many systems which allow or could allow for detection of *E. coli* O157:H7. Table 2.2 outlines the detection times and detection limits of the systems of detection discussed. Conventional methods, for example, are labor intensive and time consuming. Though conventional methods offer these set-backs they are able to guarantee the absence or presence of the organism. Immunological methods have sensitive analysis but require several possibly time consuming steps to achieve the results, which may take up to 2 days. Pathogen detection has been successful with the use of PCR. Even with PCR being successful, some draw backs include PCR offering false positives when there is more than 108 CFUs. With PCR there may still be a need for enrichment which can be time consuming. Biosensors, SPR (refer to Figure 2.4) and fiber optic, provide real-time analysis and rapid results but can also offer difficulties with rendering the technology sensitive and reliable enough. (Deisingh and Thompson 2002; 2004). In conclusion, amperometric biosensors will be evaluated as means of detecting *Escherichia coli* O157:H7. The amperometric biosensor will also be compared to current rapid detection methods (Table 2.2) in Chapter 5.

Table 2.2: Summary of methods used to detect *Escherichia coli* O157:H7
Source: Deisingh and Thompson 2003

Method	Approx. detection time	Detection limit
Plating/culturing	1 day to 1 week	Low CFUs
Biochemical tests	1 day to several days	Low CFUs
ELISA	12 h to 2 days	10–100 CFU ml^{-1}
Fluorescent bacteriophage assay	10 h	10–100 CFU ml^{-1}
Chemiluminescence enzyme immunoassay	6–8 h	10^3–10^4 cells ml^{-1}
Capillary immunoassay	7 h	0·5–1 CFU ml^{-1}
Time-resolved fluorescence immunoassay	6 h	10–100 CFU ml^{-1}
PCR	2–24 h depending on enrichment	102–105 CFU ml^{-1}
Multiplex PCR	24 h	1–2 CFU ml^{-1}
RT- PCR	6–12 h	107 CFU ml^{-1}
Laser-induced fluorescence	Few hours	Single organism
Fibre optic biosensor	*ca* 30 min	$5·2 \times 10^2$ CFU g^{-1}
SPR biosensor	1 h	5×10^7 CFU ml^{-1}
Microarrays	<1 h	55 CFU ml^{-1}
Molecular beacon	1–6 h depending on enrichment	1–10^3 CFU ml^{-1}
Integrated systems (lab-on-a-chip)	16–45 min	10^2–10^4 organisms ml^{-1}

CHAPTER 3. MATERIALS AND METHODS

This chapter contains the experimental methods, procedures, and materials used in this study to analyze the detection of *Escherichia coli* O157:H7 by an amperometric biosensor. The experiments were conducted to evaluate the usefulness of amperometric biosensor with detecting *E. coli* O157:H7. In addition, many parameters of the sensor and biological reception mechanisms were evaluated. Environmental factors affecting sensor performance were also studied. The complete testing of the system is broken up into two phases, initial testing and final testing. The initial testing contains the initial design and fabrication along with environmental probe tests. The final testing section contains the antigen-antibody testing, substrate-enzyme complex testing, and an assessment of the effect of environmental parameters on the biosensor's performance. This section outlines the procedures and analytical methods used for both the initial and final tests.

3.1 Phase 1-Initial Tests

3.1.1 Amperometric Biosensor Setup-Overview

A power source (Masteck Metered Bench Supply) was connected to the Clark Oxygen Electrode (YSI 5739 DO Probe). An Autoranging Picoammeter (Keithley Model 485) was wired to the Clark oxygen electrode and used to record the current that was generated due to changes in oxygen concentration. The fabricated outer insert was mounted to the tip of the oxygen electrode which contained the biological receptor. This configuration made up the amperometric biosensor to detect *E. coli* O157:H7 for initial testing.

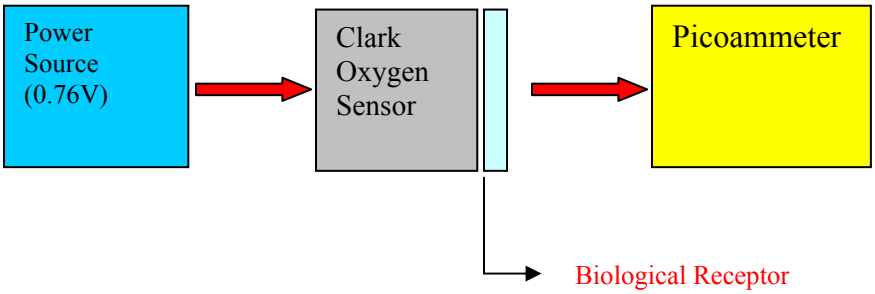

Figure 3.1: Schematic of a Bench scale Amperometric Biosensor

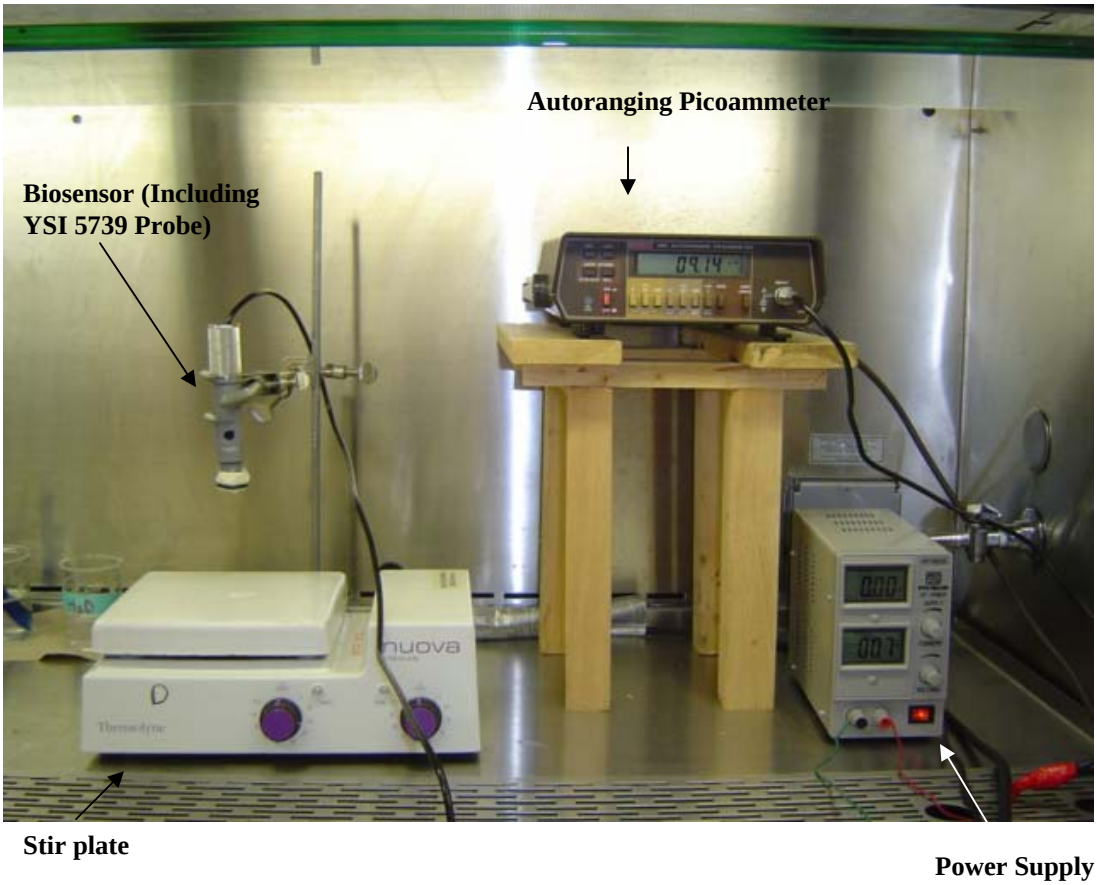

Figure 3.2: Amperometric Biosensor Setup (Phase 1)

3.1.2 Configuration of the Amperometric Biosensor for the Detection of *E. coli* O157:H7

1. External Power Source

The Mastech Metered Bench Supply was utilized to apply a potential of 0.7V DC to the DO Probe. This instrument had an output range of 0-18V DC regulated.

2. Autoranging Picoammeter

The Keithley 485 Autoranging Picoammeter was selected to detect current due to its low range of detection. The Keithley 485 can accurately detect current in the nanoamp range and has a sensitivity of 0.1pA. The Keithley 485 contains a 4.5" LCD display with front BNC connector input which was necessary for easy readout and probe connection, respectively. The maximum input potential is 30V which is well within the range needed for the Amperometric Biosensor. The Keithley 485 Autoranging Picoammeter was an affordable option that had all the necessary characteristics needed in a Picoammeter.

3. YSI 5739 DO Probe

The YSI 5739 was selected as the main oxygen sensing component of the amperometric biosensor. The YSI 5739 is a Clark type electrode consisting of a gold cathode and silver anode. The gold cathode and silver anode compose the electrolytic cell of the Clark electrode. The electrolytic cell is separated from the probing solution by an oxygen permeable membrane in this case, Teflon, which both helps to protect the electrolytic cell and to allow the oxygen to permeate. The oxygen is then reduced once a potential of 0.7V is applied in reference to the silver electrode. The reduction of the oxygen is proportional to the concentration of dissolved oxygen (in mg/L). The following equation describes this reaction (YSI Incorporated Manual).

Cathode reaction: $O_2 + 2H_2O + 4e^- \longrightarrow 4OH^-$

Anode reaction: $Ag + Cl^- \longrightarrow AgCl$

4. **Outer Insert**

The outer insert was made of polyvinyl chloride (PVC). The outer insert was designed to fit tightly onto the YSI 5739 dissolved oxygen probe including the O-ring attached to the teflon membrane while still allowing the ability for the outer insert to come on and off of the probe without any major affects to the amperometric biosensor setup. The outer insert also contains a groove for the outer O-ring which was used to secure the outer membrane, nitrocellulose. The height of the outer membrane was machined so that the inner membrane and outer membrane are able to mesh in order to negate dissolved oxygen that could have possibly entered the teflon membrane due to air pockets. The fabricated outer insert is pictured in Appendix A.

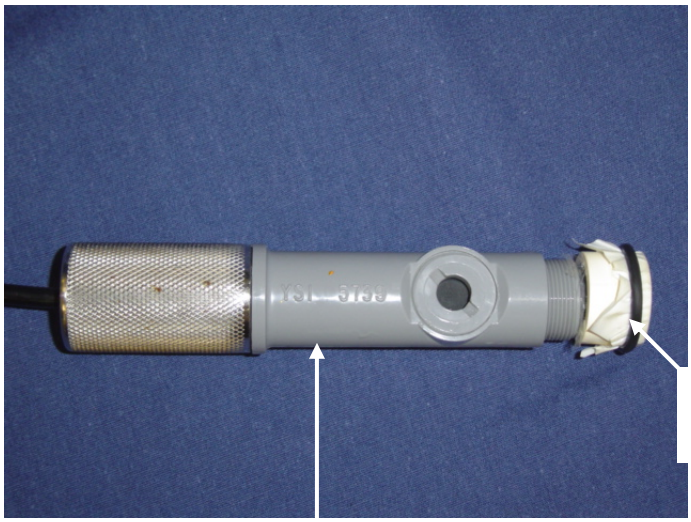

Figure 3.3: YSI Dissolved Oxygen Probe with Fabricated Outer Insert

3.1.3 Materials

- **Outer Membrane Selection- Nitrocellulose**

The outer membrane was selected based on its ability to provide good absorption properties for the immobilized antibody and a pore size that was optimum for these applications. From Muhammad-Tahir and Alocilja (2003), it was found that nitrocellulose membrane was the best material for the outer membrane. The nitrocellulose membrane in Muhammad-Tahir and Alocilja's disposable biosensor was labeled as the "capture pad".

- **Goat Anti *E. coli* O157:H7-HRP**

A conjugated goat anti *E. coli*O157:H7-HRP antibody was purchased from Fitzgerald Industrial International, Inc. The conjugated antibody was received in the lyophilized form and reconstituted with a 1ml of a fifty percent glycerol solution as instructed by Fitzgerald Industrial International, Inc Data Sheet. This allowed for a final concentration of 100μg/ml goat anti *E. coli*0157:H7-HRP solution. Once the conjugated antibody was reconstituted with the 1ml-50% glycerol solution, gentle agitation was applied to the vial for 20s. The conjugated antibody is stored at 4°C for up to 3 months.

- **Bacteria**

The heat-sterilized *E. coli* O157:H7 bacteria were ordered from Fitzgerald Industries International, Inc. The bacteria were rehydrated with phosphate buffered saline solution. A stock solution of bacteria was made at 3.5 X 10^9 cells/ml. Concentrations of *E. coli*0157:H7 bacteria were made by dilution in Phosphate Buffered Saline (PBS) solution.

- **Outer Membrane Preparation**

Various outer inserts were built using PVC material machined to fit the biosensor and allow for housing of the outer membrane. The outer insert was mounted over the tip of the dissolved oxygen sensor. The nitrocellulose was then placed over the outer insert and secured using an O-ring. The outer insert is pictured in Appendix A. Forty microliters of enzyme labeled antibody were added to the center of the attached nitrocellulose membrane. The antibody was allowed to air-dry while attached to the insert for three hours followed by 24 hour incubation at 4°C.

3.1.4 Testing of Amperometric Biosensor for the Detection of *E. coli* O157:H7

3.1.4.1 Dissolved Oxygen-Current Correlation

In order to find the correlation between dissolved oxygen concentration (mg/L) and current (amps) generated, solutions of various dissolved oxygen concentrations were prepared by adding small amounts of sodium sulfite and aerating the solutions. Sodium sulfite was utilized to reduce and deplete the dissolved oxygen concentrations in the water solution. Sodium sulfite concentrations varied in order to achieve different dissolved oxygen concentrations. A completely oxygen depleted solution was obtained by adding one gram of sodium sulfite to 500ml of distilled water as directed in the YSI 55 manual (1999). Many beakers of tap water were also aerated for various amounts of time using aquarium air pumps, tubing, and air stones. Measurements of dissolved oxygen were taken with a dissolved oxygen meter (YSI 55). The YSI 55 dissolved oxygen meter was calibrated using the instructions provided by YSI incorporated. This probe was used as a standard measurement for dissolved oxygen concentration (mg/L).

The correlation between various dissolved oxygen concentrations and current was achieved by testing the YSI 55 dissolved oxygen reading of the prepared solutions versus the current response achieved from the amperometric biosensor setup with and without the outer insert. The results were analyzed with both the dissolved oxygen meter and amperometric biosensor setup (without the biological receptor).

3.1.4.2 Variation in Current due to Change in Outer Insert

Solutions with various dissolved oxygen concentrations (mg/L) were prepared using the procedure outlined in "Dissolved Oxygen-Current Correlation". Next, the amperometric biosensor was assembled with the addition of the enzyme labeled antibody (biological receptor) to the outer insert. The amperometric biosensor probe was placed in the solutions of varying dissolved oxygen concentration and current generated was recorded. Once the current was recorded for the outer insert at each concentration of dissolved oxygen, the insert was changed and more current readings were recorded. Three outer inserts containing the biological receptor were tested and evaluated.

3.1.4.3 pH-Current Correlation

Once the amperometric biosensor was assembled, the effect pH was evaluated on the biosensor's performance. The amperometric biosensor included the outer insert which contained 40μL of 100μg/ml *E. coli* O157:H7-HRP. Four sets of beakers, one set consisting of tap water and the other three sets consisting of hydrogen peroxide were situated for testing. The four sets of beakers were adjusted to the following pH values: 5, 5.5, 6, 6.5, 7, 7.5, and 8. The pH was adjusted using a sodium hydroxide solution (approximately pH 10) and a hydrochloric acid solution (pH 1.5). Ten microliters of 1M Tris buffer was added to the beakers. The sodium hydroxide and hydrochloric acid

solutions were titrated into the beakers containing hydrogen peroxide until the corresponding pH values were reached. Once the beakers were setup, the current response of the amperometric biosensor was evaluated. The current readings were recorded after 10 minutes allowing the system enough time to reach steady state. All pH experiments were conducted and recorded in triplicate.

3.1.4.4 Antibody Concentration-Current Correlation

The outer insert was prepared by attaching nitrocellulose to the outer membrane followed by securing of the membrane to the outer insert using an O-ring. The Goat anti *E. coli* O157:H7 –HRP was prepared as noted in the Materials section of the procedure for Goat anti *E. coli* O157:H7 –HRP. Aliquots in the order of 10-60 μL were added to the nitrocellulose membrane. The antibody was allowed to air dry for three hours and then refrigerated over night. The amperometric biosensor was assembled and the outer insert with the given volume of antibody was attached. Beakers containing 28ml of 0.88M hydrogen peroxide and 40ml of distilled water at room temperature were tested by exposing the biosensor in the beaker and recording the current after steady state. Each volume of conjugated antibody was tested in triplicate. Results were recorded and analyzed.

3.1.4.5 Hydrogen Peroxide Concentration-Current Correlation

The amperometric biosensor was setup as shown in Figure3.2. Forty microliters of enzyme labeled antibody was attached to the outer insert. The biosensor was submerged in a beaker containing 40ml of distilled water. Hydrogen peroxide with a molar concentration of 0.88M was utilized. A set volume between 0-40ml of hydrogen peroxide was added to the beaker containing 40ml of distilled water. The molar

concentrations of hydrogen peroxide in the final solutions were between 0-0.404M. The experiment was conducted at room temperature (22°C). The outer insert containing the HRP labeled antibody and beaker of distilled water was changed after each amount of hydrogen peroxide was added to the beaker.

3.1.4.6 Temperature-Current Correlation

The effects of temperature on amperometric biosensor were accessed. The biosensor was assembled with 40 μL of conjugated antibody attached to the outer insert. The biosensor was submerged into solutions containing 28ml of hydrogen peroxide and 40ml of distilled water. Two sets of samples were evaluated; set one which was adjusted at pH 6.8 and another set which was not adjusted for pH. The pH for the solution that was not adjusted ranged from pH 6.2-7. Both sets of samples were adjusted to temperatures between 35°F-75°F. This was accomplished by heating samples in an Isotemp Oven until they reached the corresponding temperatures and refrigerating samples to a given temperature. Sample set one was adjusted to pH 6.8 using titrations of hydrochloric acid and sodium hydroxide. Sample set 2 was not adjusted to pH 6.8. Prior to the amperometric biosensor being utilized to evaluate current of each individual sample set, temperature readings were taken. Once the temperature of the solution was recorded, the amperometric biosensor was submerged in the sample. After 10 minutes, the time necessary for steady state, the current was recorded.

3.2 Phase 2-Final Tests

After observations were made in the initial testing phase, phase 2 of testing was implemented. The new amperometric biosensor configuration included the YSI 55 probe/meter as both the dissolved oxygen probe and meter for readout. Further details on

the instrumentation change can be found in the Chapter 4. The testing conducted is detailed in the following section.

3.2.1 Materials

- **Outer Membrane Preparation**

The outer membrane material was nitrocellulose same as used in initial testing, phase 1. The nitrocellulose was secured onto the custom fabricated insert by O-ring. Twelve microliters of antibody was applied to the nitrocellulose. The nitrocellulose was incubated at 37°C for one hour. The membranes that were not used immediately were refrigerated at 4°C overnight for no longer than one week.

- **Horseradish Peroxidase Enzyme**

The unconjugated horseradish peroxidase (HRP) was received from Biozymes Laboratories Limited. The HRP was in the form of a brown freeze-dried powder. The listed activity of the enzyme was 254 U/mg material.

- **Custom Conjugated Antibody**

The *E. coli* O157:h7 antibody was obtained from Kirkegaard & Perry Laboratories. The antibody was reconstituted at 1.0mg/ml using the HRP conjugation buffer. The antibody was conjugated with HRP using the Sure Fire Custom Conjugation kit obtained from Kirkegaard & Perry Laboratories. The Custom Conjugation consisted of a three step process. The first step consisted of adding of 100 μL of *E. coli* O157:H7 antibody to 0.3mg of activated HRP. Next, a reducing agent was added to allow conjugation of the activated HRP and antibody. Finally, a storage buffer was added to the conjugate to allow it to remain stable. An outline of the process is pictured in Figure 3.4. The complete directions for conjugation process can be found in Appendix B.

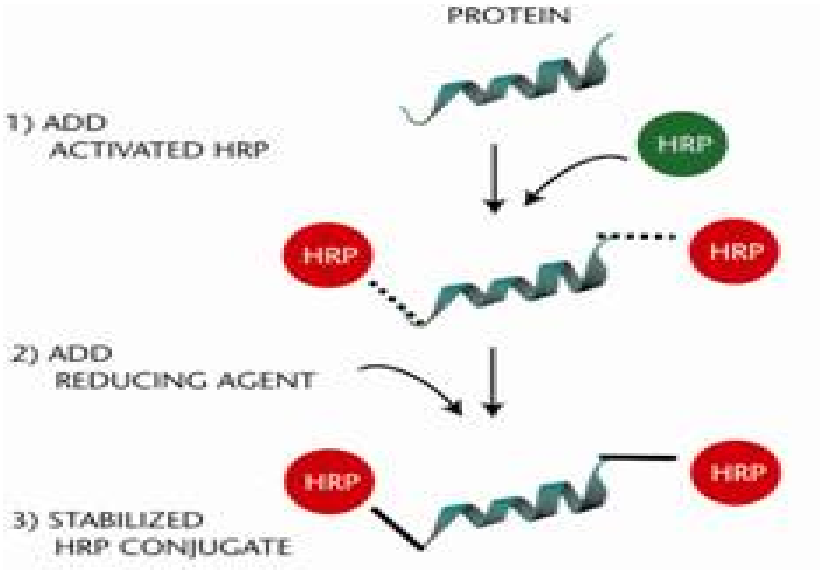

Figure 3.4: Overview of Custom Conjugation Process

- **Bacteria**

The heat-sterilized *E. coli* 0157:H7 bacteria were ordered from Fitzgerald Industries International, Inc. The bacteria were rehydrated with 1ml of a 50% glycerol solution. The rehydrated bacteria had a final concentration of 3.5 X 10^9 cell/ml. Other concentrations of *E. coli* 0157:H7 bacteria were made by serial dilution in 0.1M Tris solution.

- **Chemicals**

Other chemicals namely, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), hydrogen peroxide (3 weight percent), and buffers (Tris [pH 7], sodium citrate [pH 3,4,5] and sodium phosphate pH [6.5, 8.5]) were ordered from Sigma-Aldrich.

3.2.2 PHASE 2- Final Testing of Amperometric Biosensor for the Detection of *E. coli* O157:H7

3.2.2.1 Membrane Attachment Tests

The amount of antibody-HRP conjugate attached to the nitrocellulose membrane was compared with an antibody-HRP conjugate in solution. The purpose of this experiment was to determine if there was antibody-HRP conjugate attached to the membrane and how enzyme performance compared to the antibody-HRP conjugate in solution. This was accomplished by attaching 3μL of 1-2μg/ml of the antibody-HRP conjugate to the nitrocellulose membrane utilizing the same procedure noted for preparation of the outer insert. The nitrocellose membrane was washed in a 0.1M tris solution. The nitrocellulose membrane with attached antibody-HRP conjugate was placed in a vial. Next, 0.5ml 3,3′,5,5′-Tetramethylbenzidine (TMB) liquid substrate was added to the vial. After ten minutes, 0.5ml of hydrochloric acid was added to stop the colorimetric reaction. The same procedure was repeated with the antibody-HRP conjugate in solution (unattached to the nitrocellulose membrane). This solution contained the same concentration of antibody tested on the nitrocellulose membrane. The colorimetric change was quantified by taking absorbance readings at 450nm using a spectrophotometer. The colorimetric change was also quantified for a nitrocellulose membrane which contained 1ml of Tween followed by addition of the antibody-HRP conjugate. The nitrocellulose membrane with Tween was used as a control in this experiment because Tween acts to block the binding sites found on the nitrocellulose membrane. A membrane with 3μL of 2μg/ml antibody-HRP conjugate followed by

addition of 1ml of Tween was also evaluated for colorimetric change with the use of TMB substrate system.

3.2.2.2 Unconjugated and Conjugated HRP Testing

A stock solution of horseradish peroxidase (HRP) enzyme was prepared in distilled water and 0.1M tris buffer. A substrate consisting of distilled water, 0.001M ABTS, and 0.005M hydrogen peroxide, and tris buffer was prepared. Another substrate which consisted of 0.005M hydrogen peroxide and tris buffer was also prepared. The HRP was added to the substrate consisting of ABTS and hydrogen peroxide. HRP was also added to the substrate consisting of hydrogen peroxide and tris buffer only. The total solution volume with the addition of HRP for all experiments was 6ml. Using the YSI 55 Dissolved Oxygen probe and meter the dissolved oxygen concentration was monitored at steady state for the substrate (t = 0) then for ten minutes beginning with the point of enzyme inoculation. All experiments were performed in triplicate.

The same procedure was incorporated using the commercial and custom conjugated antibody-HRP. The amount of antibody-HRP that was added to the substrate was based on the HRP molarity. All commercial and custom conjugated antibodies were tested with substrate system which contained ABTS. Final concentrations of HRP were in the same order as the unconjugated antibody (10^{-8}M).

3.2.2.3 Temperature Testing

The effect of temperature on the amperometric biosensor's performance was assessed utilizing the range of temperatures commonly found in southern Louisiana. The biosensor was submerged into the substrate solutions containing 0.005M hydrogen peroxide, 0.001M ABTS, 0.1M tris buffer, and distilled water. The substrates were

adjusted to temperatures ranging between 4-40°C. This was accomplished by heating samples in an Isotemp Oven and incubator until they reached the corresponding temperatures and refrigerating samples to a given temperature. Prior to the amperometric biosensor being utilized to evaluate dissolved oxygen of each individual sample set, temperature readings were taken. Once the temperature of the beaker was recorded, the amperometric biosensor with attached conjugated antibody at a concentration of 6×10^{-8}M was submerged in the sample. Dissolved oxygen readings were taken every thirty seconds for ten minutes. All readings for a given temperature were evaluated in triplicate.

3.2.2.4 pH Testing

Substrates were prepared with buffers (Tris [pH 7], sodium citrate [pH 3,4,5] and sodium phosphate pH [6.5, 8.5]), 0.001M ABTS, and 0.005M hydrogen peroxide. The final pH in each substrate was evaluated using an Orion pH meter. The dissolved oxygen concentration was evaluated by exposing the YSI 55 probe with outer insert into substrates. The dissolved oxygen concentration was monitored over a 10 minute time period. All tests were performed in triplicate. The amperometric biosensor's performance at different pH values was also an evaluation of the enzyme substrate interaction and product formation ability at different pH values.

3.2.2.5 Escherichia Coli O157:h7 Testing

Bacterial concentrations 1-5000 cells/ml were prepared in distilled water and tris buffer. The outer insert was attached to the YSI 55 probe. The YSI probe with insert was submerged into the beaker of a given bacterial concentration for 5 minutes. The probe and insert were then removed and placed into a beaker containing 0.1M tris buffer

at a final concentration of 20ml. The probe and insert were allowed to remain in the wash for two minutes, after which the probe was submerged into a beaker containing 0.001M ABTS and distilled water (final volume 6ml). After steady state reading, dissolved oxygen was recorded (t = 0). Hydrogen peroxide was added to obtain a 0.005M final concentration at the final volume of 6ml. Simultaneously, dissolved oxygen (mg/L) readings were recorded every 30 seconds for 10 minutes. This procedure was repeated for every bacterial concentration tested in triplicate. The procedure is outlined below:

1. Outer insert applied to dissolved oxygen sensor
2. Sensor with outer insert exposed into bacteria for 5 minutes (A, Figure 3.5)
3. Sensor with outer insert submerged into wash solution for 2 minutes (B, Figure 3.5)
4. Sensor with outer insert submerged into ABTS/distilled water (C, Figure 3.5)
5. Dissolved Oxygen Reading recorded (t = 0)
6. Hydrogen Peroxide added to ABTS/distilled water
7. Dissolved Oxygen readings recorded every 30s for 10 minutes.

3.2.2.6 *E. coli* O157:H7 Testing with the use of Two Antibodies

The amperometric biosensor was analyzed with the use of a sandwich antibody assay. First, ten microliters of an unlabeled antibody for *E . coli* O157:H7 was applied to the outer insert with washed nitrocellulose membrane. The antibody was incubated at 37°C for 1 hour. Next, thirty microliters of Tween was applied to the membrane. Then, the membrane was washed with a 0.1M tris buffer solution (pH 7).

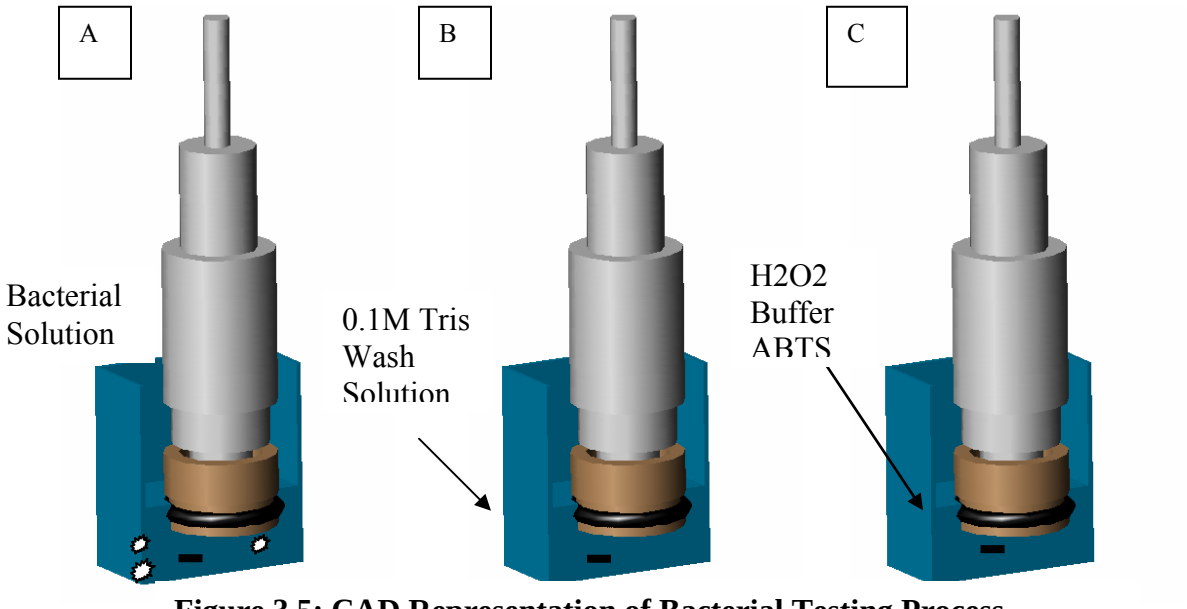

Figure 3.5: CAD Representation of Bacterial Testing Process

The outer insert was applied to the YSI probe and exposed to the bacterial solution. The membrane was washed yet again with a 0.1M tris solution. Sixteen microliters of the conjugated HRP-*E. coli* antibody was applied to membrane. The outer insert was applied to the YSI probe and washed with 0.1M Tween solution. Finally, the amperometric biosensor was exposed to substrate and the sensing procedure with the use of one conjugated antibody was followed.

CHAPTER 4. INITIAL PHASE RESULTS AND DISCUSSION

The following chapter outlines the results obtained during the initial phase of testing of an amperometric biosensor to detect *E. coli* O157:H7. The goal of this project is to test the feasibility of utilizing the amperometric sensing technology to detect the *Escherichia coli* O157:H7 in ground and surface water. The targeted area for this project is Lake Pontchartrain in southern Louisiana. The initial phase is characterized by utilizing the setup pictured in Chapter 3, Figure 3.2 for all results discussed in this section. The purpose of the initial phase is to optimize the experimental design and testing phases of the final amperometric biosensor system. The chapter allows a basis for planning the parameters explored in the final phase of testing the amperometric biosensor, Chapter 5. All results discussed were considered in the final design.

4.1 Current -Dissolved Oxygen

Figures 4.1-4.3 illustrate the relationship between current and dissolved oxygen for the initial amperometric biosensor set-up. It was important to note the relationship between current and dissolved oxygen mainly for converting from the readout displayed on the picoammeter (A) to the dissolved oxygen (mg/L) concentration in the liquid. Figure 4.1 displays the relationship of current to dissolved oxygen with and without the use of an outer insert. This relationship is outlined by the expression

$$y = 1.4448x - 0.3013 \qquad \text{Equation 3.1}$$

Where y = Current (μA) and x = Dissolved Oxygen Concentration (mg/L).

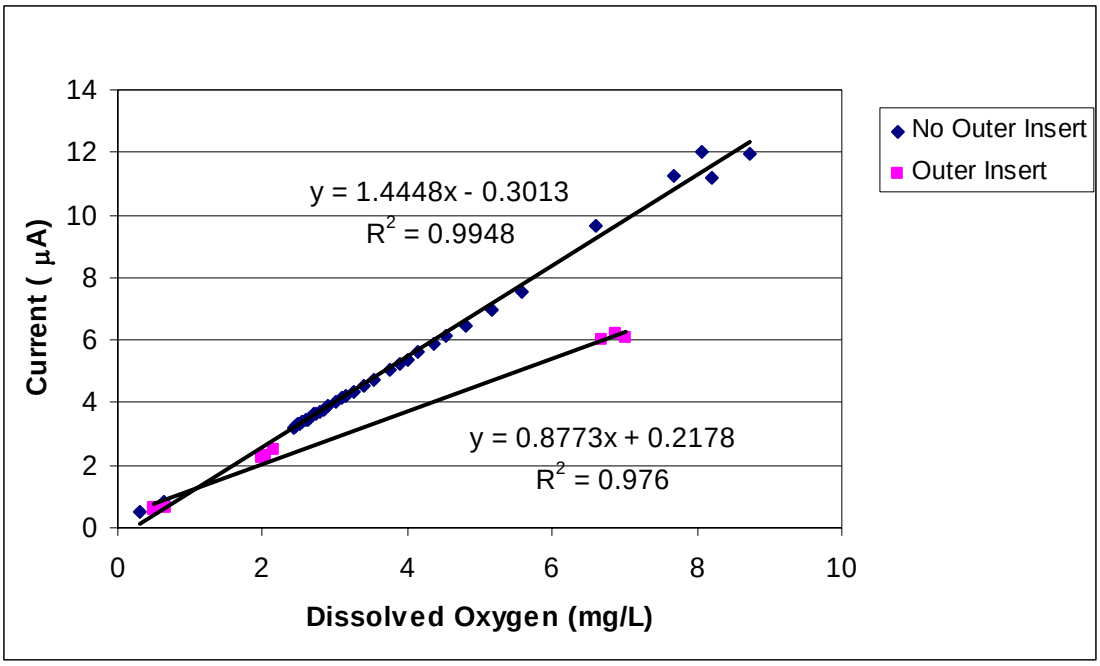

Figure 4.1: Current versus Dissolved Oxygen for Amperometric Biosensor Setup

It is also important to describe the relationship between current (A) and dissolved oxygen (mg/L) for the amperometric biosensor setup with the use of the outer insert. Figures 4.2-4.3b shows the relationship of current to dissolved oxygen for the phase 1 amperometric biosensor setup. Figures 4.3a-b illustrate the equations obtained utilizing three outer inserts denoted by outer insert 1, outer insert 2, and outer insert 3. The equation describing the amperometric biosensor with three outer inserts:

$$y = 0.8773x + 0.2178 \qquad \text{Equation 4.2}$$

Where y = Current (μA) and x = Dissolved Oxygen (mg/L)

The equation found with the use a single outer insert:

$$y = 0.8467x + 0.3375 \qquad \text{Equation 4.3}$$

In both set of equations describing the current to dissolved oxygen ratios (slopes in Equation 4.2 and 4.3) obtained from the amperometric biosensor setup with outer

insert, the relationship of current to dissolved oxygen is similar. Also, it can also be noted that the ratio (slopes in Figure 4.1, with and without outer insert) of current to dissolved oxygen in the amperometric biosensor with outer insert is decreased from that of the sensor without the outer insert. This may be contributed to decreased oxygen diffusion due to increased membrane layers. In other words, oxygen gas has to diffuse through two layers of membranes, teflon and nitrocellulose, instead of the one layer, teflon, as seen with the amperometric biosensor without the outer insert. This ratio could be lowered with the use of conjugated antibody, which would provide for a larger diffusion layer for oxygen, therefore decreasing the diffusion rate.

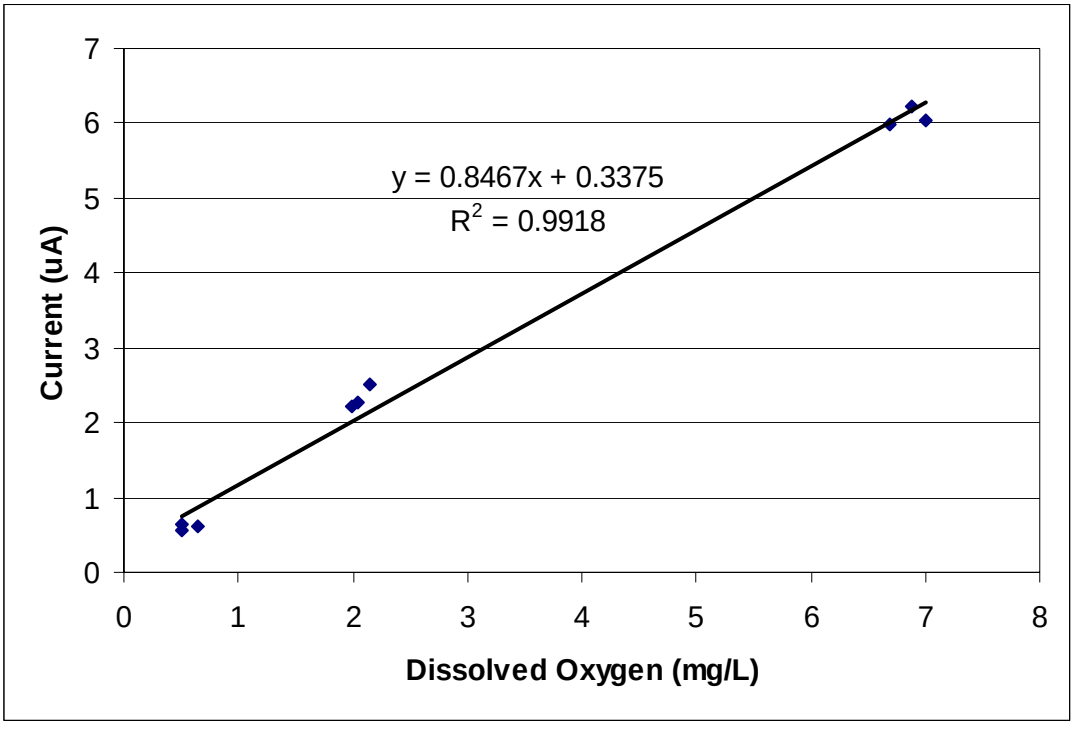

Figure 4.2: Current versus Dissolved Oxygen for Amperometric Biosensor Setup with the use of One Outer Insert (Phase 1)

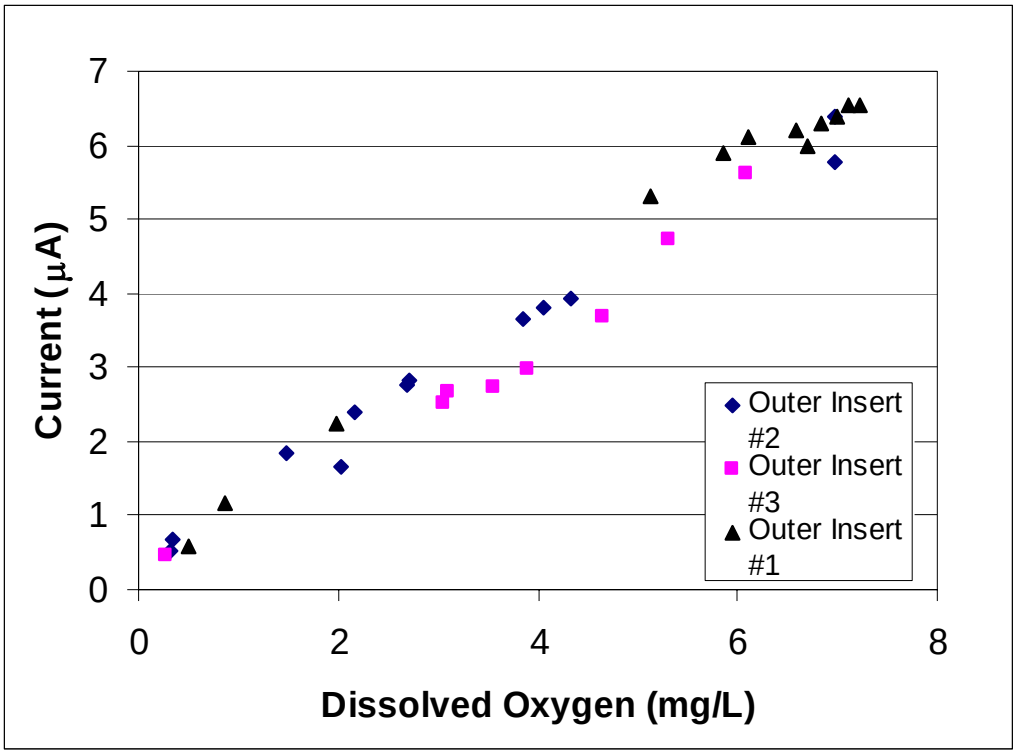

Figure 4.3a: Current versus Dissolved Oxygen for Amperometric Biosensor Setup with the use of Various Outer Inserts (Phase 1)

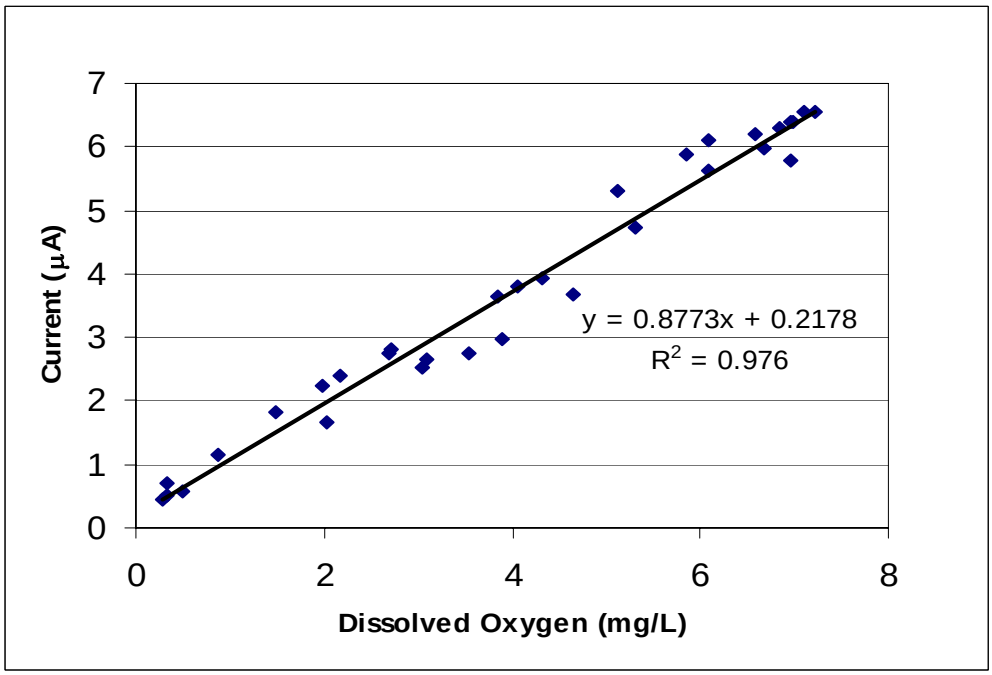

Figure 4.3b: Current versus Dissolved Oxygen for Amperometric Biosensor Setup with the use of Pooled Data of Various Outer Inserts (Phase 1)

Figure 4.3a-b allows further insight to the analysis of the behavior of the amperometric biosensor setup. For the original, phase 1, amperometric biosensor testing setup, there is a need to change the outer insert when testing water samples or samples in the laboratory. The need to change the outer insert will follow into the final phase testing of the amperometric biosensor. Figure 4.3a-b also shows that with the changing of outer inserts, the current signal follows the same trend as seen with one outer insert at different dissolved oxygen concentrations. This can be seen in comparing Figure 4.2 to 4.3a-b. Equations 4.2 and 4.3 further characterize this relationship, with the slope for one outer insert and the slope of the collaboration of outer inserts being 0.8467 and 0.8773 respectively. From this it can be concluded that very little variation in current occurs as a result of changing outer inserts. Ultimately, it was assumed that little variation is occurring in the amperometric biosensor system due to changing of the outer insert.

4.2 Substrate Concentration

The first set of tests in the initial phase was conducted to evaluate the effect of substrate concentration on the biosensor's performance, which in this system consisted of concentrations of hydrogen peroxide. Figure 4.4 shows the trials conducted with the testing of hydrogen peroxide. In the experiment very little signal was generated with the use of hydrogen peroxide and the commercially available conjugated HRP-*E.coli* antibody. The procedure for these tests can be found in the Chapter 3, the Methodology under the initial testing Phase 1-Hydrogen Peroxide Concentration. It is important to note that a 40 microliter volume of conjugated antibody was utilized and all readings were taken after 10 minutes, which allowed enough time for the system to reach steady state. In trial 1, which tested hydrogen peroxide volumes between 0 to 15ml added to

solution (0-0.228M hydrogen peroxide), a change in current of only 1.07μA was seen. A change in 1.07μA in current would correspond to a change in dissolved oxygen at 22 °C of 0.87mg/L using equation 4.2. The next two trials of hydrogen peroxide concentration testing show similar trends. In trial 2, the change in current for 15ml and 30ml hydrogen peroxide added to the system (0.228M and 0.377M) were 0.66μA and 1.13μA respectively. These values correspond to a 0.38mg/L change in dissolved oxygen for 15ml and 0.94mg/L for 30ml hydrogen peroxide added to solution, all of which were measured at 22 °C. The third trial showed a 0.3mg/L change in dissolved oxygen for 15ml (0.228M hydrogen peroxide) and 0.61mg/L with 30ml of hydrogen peroxide added to system (0.377M hydrogen peroxide). After analyzing all trials, the maximum change in dissolved oxygen achieved for 15ml of hydrogen peroxide added to system was 0.86 mg/L and at 30ml of hydrogen peroxide added was 0.94mg/L.

There was a vast amount of variation in dissolved oxygen production between trials. Small increases in dissolved oxygen concentration may have contributed to the variation. This can cause problems in the system for many reasons. One reason is that the small change in dissolved oxygen can not be easily distinguished from small increases in oxygen due to diffusion of oxygen from air surrounding the sample. Secondly, there is an extremely large amount of the substrate, hydrogen peroxide in the system. Large amounts of hydrogen peroxide over a given period of time are known to cause cell death. Although all tests were conducted utilized heat sterilized *E. coli*, cell death could be a major concern for the system especially when cell rupture occurs. This could result in cell fragments, which may not be distinguishable from whole cells, in the future possibly

leading to increased bacterial detection. This could be an example of a false positive for the amperometric biosensor.

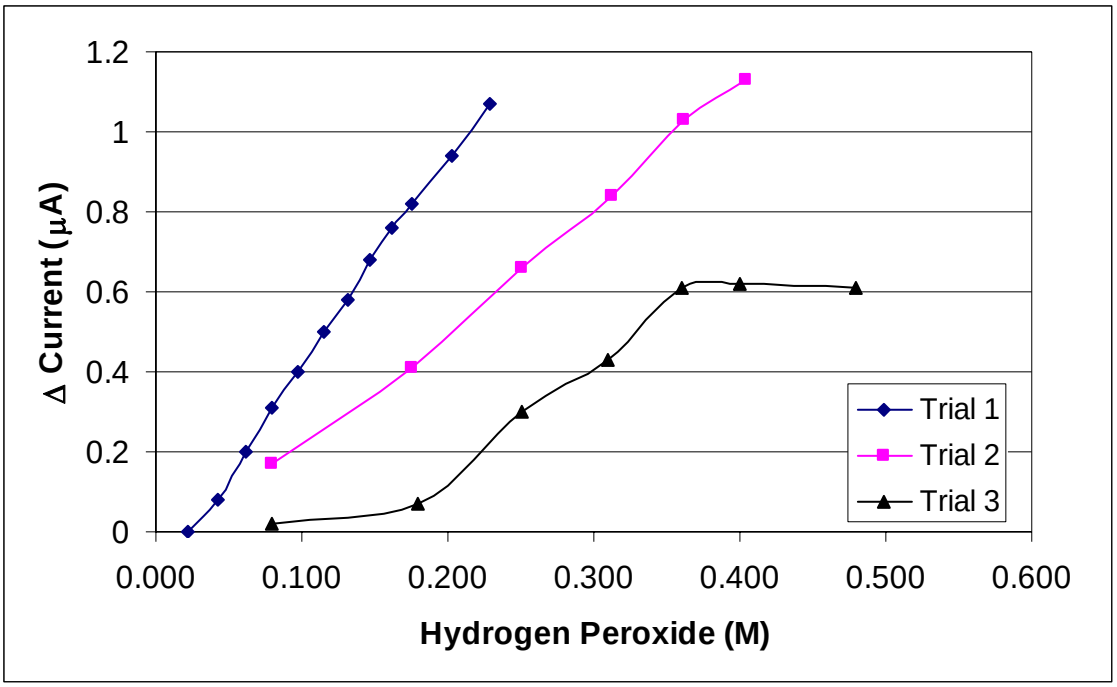

Figure 4.4: Current versus Hydrogen Peroxide

From phase 1, amperometric biosensor testing with varying substrate concentrations, it can be assumed that with the use of the commercially available conjugated HRP-*E. coli* antibody and substrate hydrogen peroxide, a significant change in dissolved oxygen concentration and/or current was not achieved. A significant change in dissolved oxygen was identified as one that has at least a 3mg/L increase in dissolved oxygen. This would allow a distinction between natural increases in dissolved oxygen due to diffusion from the outside environment and increases in dissolved oxygen due to oxygen production from the binding of enzyme and substrate. The use of an oxidizing agent or another substrate-enzyme system that produces oxygen may be necessary for the next phase of testing. However, with the use of the hydrogen peroxide and HRP

conjugated antibody used in this experiment, no significant signal (less than 3 mg/L) with binding of enzyme and substrate or increase in dissolved oxygen can be detected.

4.3 Antibody Volume

The antibody volumes of 0-60μL were evaluated. The results can be found in Appendix C. The three trials displayed different behavior patterns. The first trial reached maximum current at 30μL and peaked through 60μL. On the other hand, trials 2 and 3 reached maximum current at 40μL, and decreased at 60μL. All three trials distributed very large increases in dissolved oxygen concentrations which did not agree with data achieved from hydrogen peroxide and conjugated antibody testing nor did it agree with literature on dissolved oxygen production between hydrogen peroxide and horseradish peroxidase (Hernandez-Ruiz et al. 2001). The behavior distributed by varying antibody concentration with a set volume of hydrogen peroxide is inconclusive at this point. Since dissolved oxygen concentration profiles over time were not conducted in these tests, the large oxygen increase can not be justified nor does it model the behavior previously seen by the HRP and hydrogen peroxide.

4.4 Current –pH

Figures 4.5a-b displays the results seen at various pH values with the amperometric biosensor in Phase 1. Figure 4.5a shows a comparison of current obtained using the control, distilled water and the HRP-*E. coli* antibody. This control models what current would result at a given pH if no substrate were present. The three trials with the use of the substrate, hydrogen peroxide, were compared to this data. From figure 4.5b, the difference in current between the control and the use of hydrogen peroxide can be seen. This is denoted as ΔCurrent. The change in dissolved oxygen was calculated from

the ΔCurrent values using Equation 4.2. From table 4.2, the largest increase in current and dissolved oxygen occurred at pH 7. From Figure 4.5b, the optimum range of pH for this system would be at pH values 6.5 -7. An acceptable range in signal would be between pH 6-8. The lowest average signal was seen at pH values 5-5.5. The change in current was approximately half of which was seen at pH 6-8. These values agree with documented literature which found highest oxygen production achieved with hydrogen peroxide and HRP at pH 6.5-8 (Hernandez-Ruiz et al, 2001).

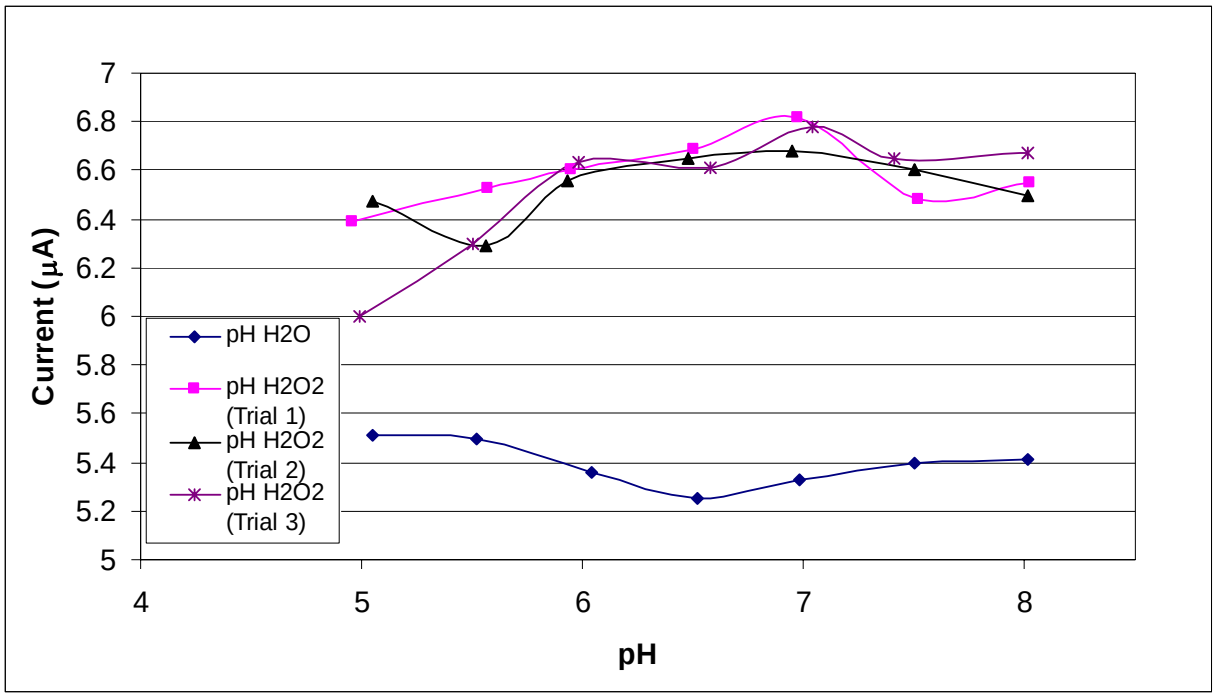

Figure 4.5a: Current versus pH-Phase 1 Testing

4.5 *E. coli* O157:H7 Concentrations

Preliminary bacterial testing was conducted in Phase 1 and results are pictured in Figures 4.6a-b. Since there was no significant signal generated with conjugated HRP-*E. coli* antibody and hydrogen peroxide, the addition of bacteria did not create a notable trend. In this study, a significant signal is an increase in dissolved oxygen of 3mg/L, which would ideally give a signal that can be differentiated from natural increases in dissolved oxygen.

It seems from these figures, that increasing bacterial concentration could either increase or decrease dissolved oxygen levels.

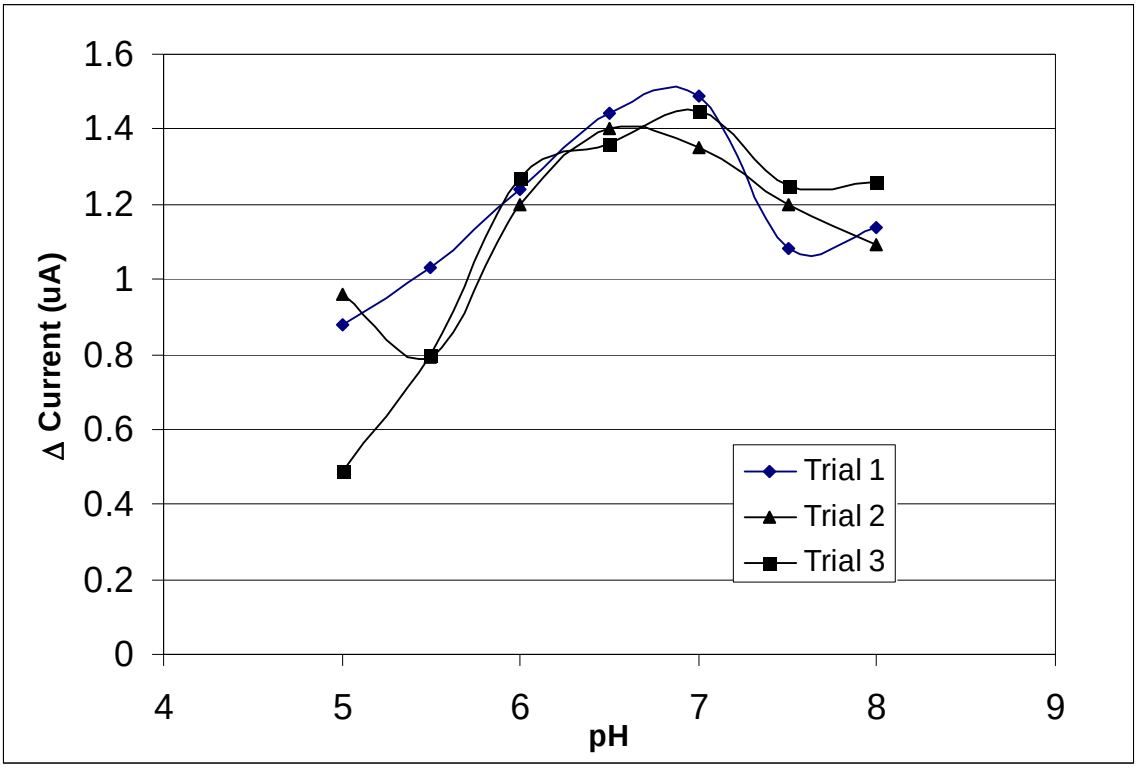

Figure 4.5b: Current versus pH-Phase 1 Testing

Without a behavior profile for the HRP- *E.coli* antibody and hydrogen peroxide, in other words no distinguishable signal, *E. coli* concentrations profiles can not be compared.

4.6 Conclusions

The initial phase, phase 1 testing brought some very important results that would need to be addressed in Phase 2-Final Testing. The current signal, with the use of the outer insert and without the use of the outer insert, was proportional to dissolved oxygen concentration. One important point to evaluate is the dissolved oxygen production with HRP-*E. coli* and hydrogen peroxide. Current response and corresponding change in dissolved oxygen were evaluated for concentrations up to 0.377M of hydrogen peroxide

for the conjugated antibody. The 0.377M hydrogen peroxide only allowed for a change in dissolved oxygen of 0.94mg/L. This was not easily distinguishable from possible diffusion of oxygen from the environment. Therefore a need for another substrate or an agent to enhance oxygen production would be necessary. Some changes in the bacterial sensing methodology are necessary to ensure that the oxygen production is obtained from the binding of the antigen to the antibody and interaction of conjugated HRP to horseradish peroxidase. At its present state, the amperometric biosensor can not detect bacterial cells. The effect pH on the biosensor's performance was evaluated for this system. The optimum range of pH was 6-8, which closely resembles the behavior of hydrogen peroxide and HRP (free in solution) found in literature. The amperometric biosensor system also showed a high degree of linearity between current and dissolved oxygen at 22°C and pH 6.8 (Figure 4.2, $R^2 = 0.99$). Since the system would require a change in outer insert with each testing application, the variation in changing outer inserts was addressed. It was concluded that changes in dissolved oxygen were not due to variations in the fabricated outer insert.

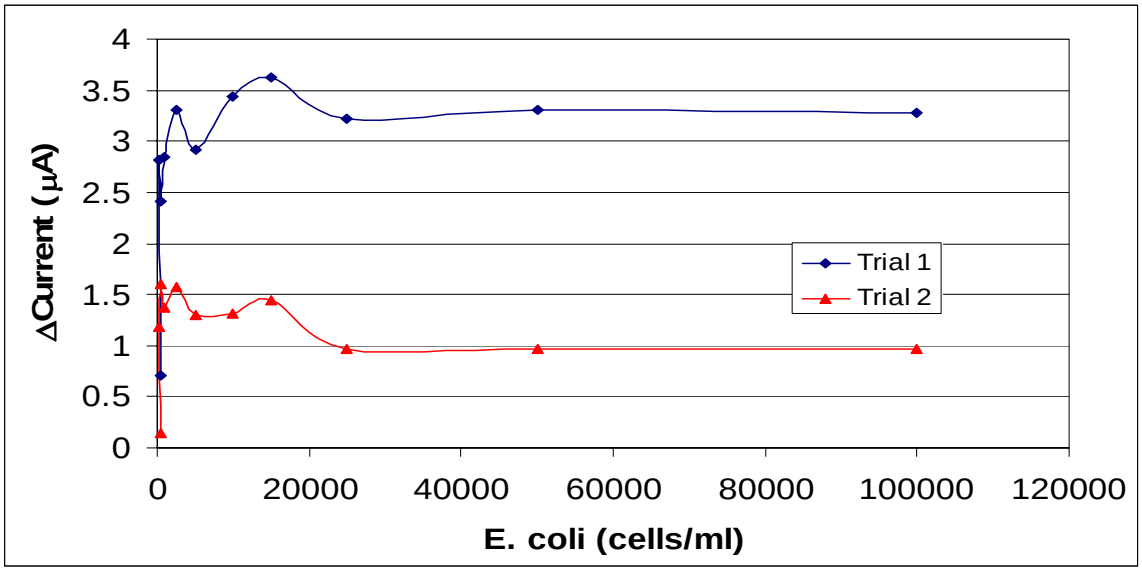

Figure 4.6a: E.coli O157:H7 concentration versus Current

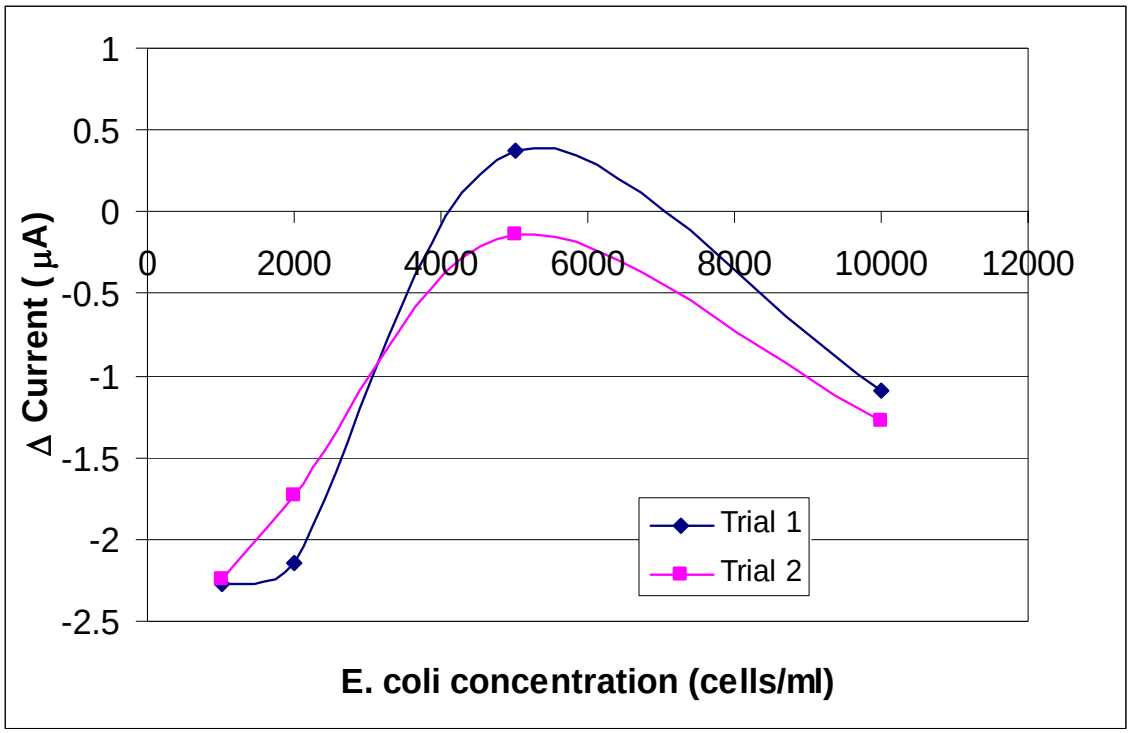

Figure 4.6b: E.coli O157:H7 concentration versus Current

CHAPTER 5. FINAL PHASE RESULTS AND DISCUSSION

The purpose of this chapter is to further examine the use of the amperometric biosensor to detect *Escherichia coli* O157:H7. After the Initial Phase testing, some changes occurred in the methodology to address the problems involved in the system. There were changes made to the testing apparatus as well as bacterial testing methodology. A YSI 55 dissolved oxygen probe and meter with attached outer insert (refer to Methodology-Final Phase) were used in these experiments to test dissolved oxygen concentrations. Dissolved oxygen profiles were recorded over time to allow more insight to what occurred during the ten minute time period. The reagent 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was introduced into the system to enhance the rate of oxygen production. Barr and Aust (1993) found that the production of oxygen with hydrogen peroxide and horseradish peroxidase was greatly increased with the use of ABTS which is oxidized to cation radicals by the peroxidase. Experiments were conducted to compare oxygen production with the use of horseradish peroxidase (HRP) enzymes from different sources, both conjugated an unconjugated. The testing was conducted by utilizing ABTS with hydrogen peroxide and a form of HRP (conjugated or unconjugated). The effect of environmental factors, namely the effect of the pH and temperature, on the biosensor's performance was also evaluated for the new system. The amperometric biosensor's ability to detect bacterial cells utilizing *E. coli* O157:H7 was analyzed.

5.1 Membrane Attachment

The first experiment conducted to transition from the Initial Phase to Final Phase of testing was the membrane attachment experiment. Since small current signals were generated from small increases in dissolved oxygen during the Initial Phase of testing, it was necessary to determine if the low rate of oxygen production could be contributed to an absence of enzyme on the nitrocellulose membrane of the outer insert. The enzyme in the initial phase was conjugated to antibody by the supplier. Therefore, the absence of enzyme activity may suggest that the conjugated antibody was not present on the membrane. This would possibly signify that no attachment was seen between the commercially conjugated antibody and nitrocellulose membrane. In order to test this premise, the antibody was attached to the membrane using the procedure outlined in the Methodology. Once the HRP conjugated *E. coli* antibody was attached, the substrate 3,3′,5,5′-Tetramethylbenzidine (TMB) was added to the membrane. TMB is commonly used with HRP-conjugated antibodies for many applications. A colorimetric reaction is observed with the interaction of the substrate TMB and horseradish peroxidase (HRP) (Croci et al, 2001). The interaction of TMB with the attached conjugated HRP-*E. coli* antibody on the membrane was compared with the reaction of TMB and the HRP-*E. coli* conjugated antibody in solution. The complete procedure can be found in the Methodology Section- Chapter 3.

Figure 5.1 shows the results obtained from the membrane attachment testing. The absorbance measurement of the membrane was found by placing the membrane with attached conjugated HRP- *E. coli* antibody at the bottom of a vial and adding the substrate and stop solution to the vial followed by absorbance readings. In contrast, the

HRP conjugated antibody in solution was directly added to the vial followed by substrate, stop solution, and absorbance readings. The difference in these measurements is denoted in Figure 5.1 by "S" for solution and "M" for membrane. When 1 μg/ml of conjugated HRP-E.coli antibody was used, an absorbance of 0.491AU was observed in solution while 0.543AU was observed on the membrane. When 2μg/ml conjugated antibody was used in solution and attached to the membrane, average absorbance readings were 0.785 and 0.721AU, respectively. In order to compare with a membrane which had blocked sites, Tween was applied and then 1 μg/ml conjugated antibody was added. On Figure 5.1, this is denoted as "3M", which has an absorbance of 0.024AU. Label "4M" represents the adding of conjugated HRP-*E. coli* antibody followed by the addition of Tween to block sites on the nitrocellulose which do not have conjugated HRP-antibody attached. The average absorbance seen from this phenomenon is 0.381AU.

The average absorbance values for the 1μg/ml conjugated antibody, both attached to the nitrocellulose membrane and in solution, were within 90% of each other. A similar trend was seen in the 2μg/ml conjugated antibody. This can be compared with the absorbance reading of the control, the membrane in which binding sites were blocked with Tween, which had an absorbance value close to 0. It was concluded from these results that the absorbance readings detected from the interaction of TMB and the conjugated HRP, both attached and in solution, were similar. Also, very little colorimetric change occurred as a result of binding sites being blocked by Tween. This experiment indicates that the membrane did contain the attached conjugated HRP-*E. coli* antibody and the minimal oxygen production seen in Phase 1 can not be due to absence of HRP attachment to the nitrocellulose membrane.

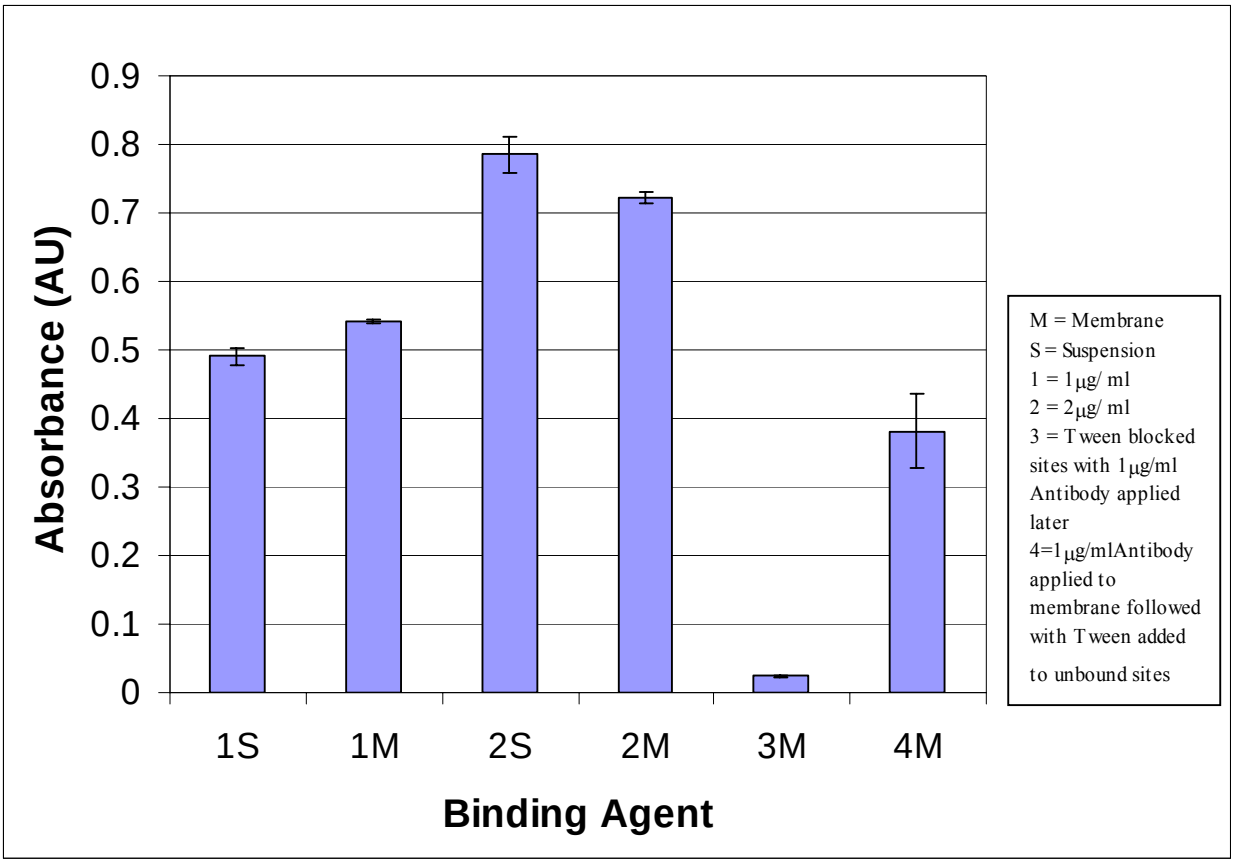

Figure 5.1: Absorbance Readings of TMB and HRP conjugated *E. coli* Antibody

5.2 Dissolved Oxygen Concentrations

After determining that the outer insert contained an attached conjugated HRP-antibody, it was necessary to identify oxygen production due to the reaction of HRP and hydrogen peroxide. This experiment consisted of the amperometric biosensor with attached HRP- *E.coli* antibody and beakers containing distilled water. The baseline dissolved oxygen concentration, or change in dissolved oxygen seen without the substrate, is shown on the Figure 5.2.

On Figure 5.2, the average dissolved oxygen production is shown in a ten minute time period. From this graph, you can see that over a ten minute period with and without the use of stirring at minimal speed, there is a reduction in oxygen. This may be contributed to consumption of oxygen by the electrode. The effects of oxygen

consumption by the electrode are greater without stirring than with stirring. No clear conclusions can be derived from this study, although possible explanations are mentioned. The higher drop (without stirring) may be due to the formation of a localized boundary layer near the electrode. The lower dissolved oxygen drop in the stirred versus unstirred sample may be due to increased aeration.

5.3 Effect of HRP Concentration and Hydrogen Peroxide

The reaction between HRP (free enzyme) and hydrogen peroxide was studied in in order to determine its affects on oxygen production. The need for an oxygen enhancer was determined based upon these results. Figure 5.3 shows the results obtained from testing various concentrations of HRP in solution with 5mM of hydrogen peroxide. The range of HRP concentration tested was determined from work done by Hernandez-Ruiz et al (2001). In their studies, the HRP enzyme concentrations in the range of 0.5-0.1μM were examined with the use of 5mM of hydrogen peroxide.

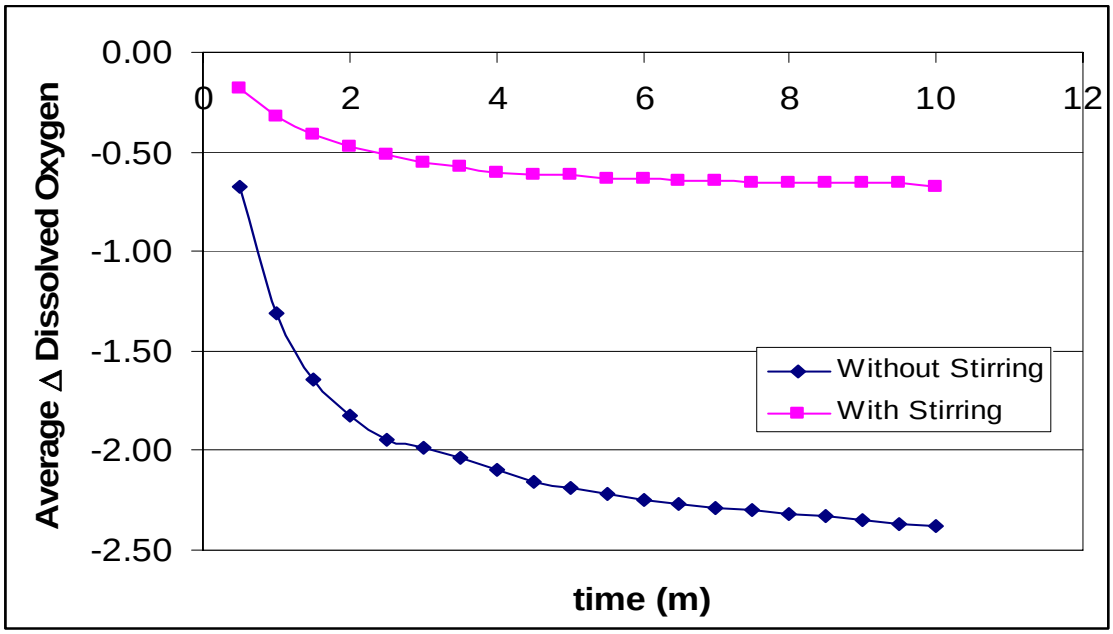

Figure 5.2: Dissolved Oxygen versus Time

From Figure 5.3, the trend between HRP concentration and hydrogen peroxide concentration can be examined. The curves of dissolved oxygen production versus time are usually hyperbolic (Hernandez-Ruiz et al, 2001). Though the curve of HRP versus hydrogen peroxide should be hyperbolic over ten minutes, the hyperbolic curve was observed only at 0.6×10^{-6}M HRP.

Since the goal for the amperometric biosensor was to detect bacterial cells within 10 minutes, the experiment was terminated at this point. Also, from Figure 5.3, we can see that the higher the concentration of HRP, the higher the dissolved oxygen production. This is consistent with the findings from Hernandez-Ruiz et al (2001). However Hernandez-Ruiz and coworkers found that the initial rate of oxygen production to increase with increased HRP concentration when a constant concentration of hydrogen peroxide was used. This is consistent with our finding in this research except for values lower than 0.2μM HRP. This could have been due to inaccuracy (±0.1mg/L) of the meter and the small amount of oxygen production achieved at these concentrations.

The minimum volume of solution needed for accurate testing of the new amperometric biosensor system is 6ml. The minimum volume was determined by adding 1ml volume of water in a beaker and adding the stir bar until a dissolved oxygen readings was able to be measured. Six milliliters was determined to be the minimum volume for this system. Therefore, there is a great need to reduce the amount of antibody used in one application. Ideally, 1ml of antibody at 6×10^{-8}M would be able to last for at least 10 applications (outer inserts). In order to do so, a lower concentration than 0.1μM HRP would be necessary. At the same time lowering the final concentration under 0.1μM did not allow a distinguishable change in dissolved oxygen concentration. Hence, a reagent

that would increase the rate of oxygen production would be necessary to add to the amperometric biosensor. The reagent 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was chosen to enhance oxygen production. Barr and Rust (1993) found ABTS dramatically increased the rate of oxygen evolution with the use of horseradish peroxidase and hydrogen peroxide.

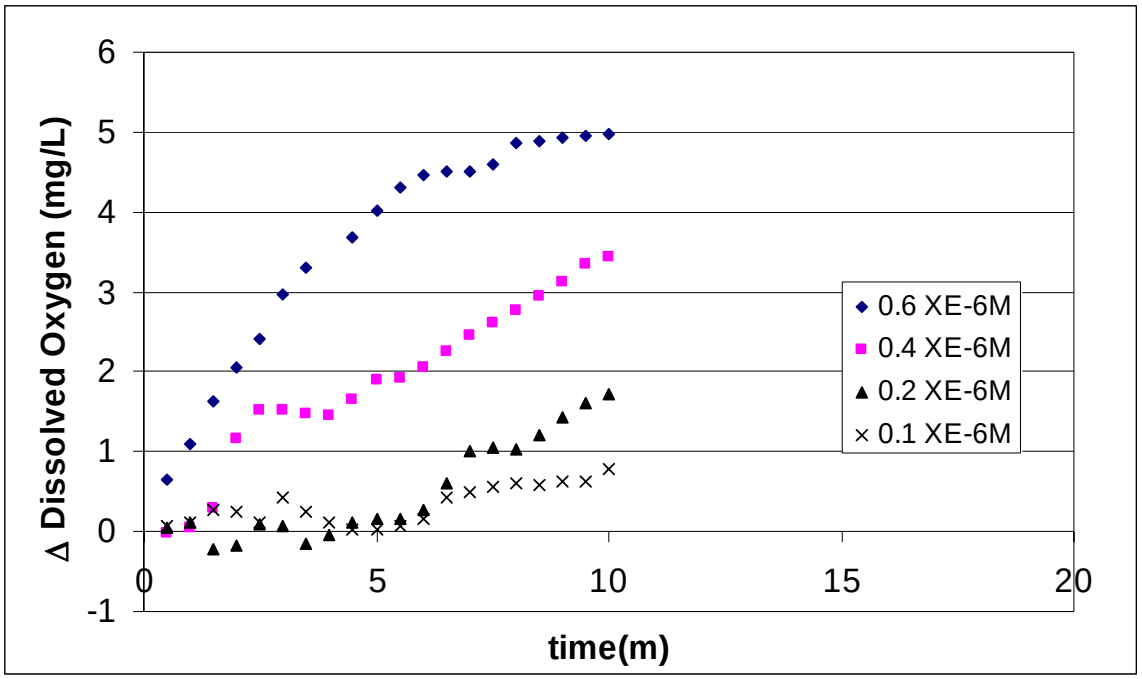

Figure 5.3: Change in Dissolved Oxygen Production at Various Horseradish Peroxidase Concentrations

Figure 5.4 shows the results found with the use of HRP-C, hydrogen peroxide, and ABTS in solution. Since ABTS increased oxygen production, the concentration of HRP could be decreased to achieve a desired increase in oxygen concentration. The reduced HRP requirement relates to reduced HRP conjugated antibody, hence lowered per sample cost. It can be noted from Figure 5.4 that with the use of ABTS, a higher dissolved oxygen production is achieved with lower concentration of HRP-C than with

hydrogen peroxide alone. However, further testing of conjugated HRP enzyme activity was conducted to evaluate oxygen production with the use of ABTS.

Compounds like ABTS and chlorpromazine (CPZ) are oxidized to cation radicals by horseradish peroxidase. It was found that the presence of ABTS as a reductant for HRP dramatically enhanced oxygen production and is also dependent upon hydrogen peroxide concentration. From this study, the rate of oxygen evolution with the use of ABTS, hydrogen peroxide, and HRP, was first order. The rate constant 1.1 $M^{-1}s^{-1}$ was calculated in this study. The results suggested that oxygen production catalyzed by peroxidases is dependent upon a compound, like ABTS, which is oxidized by peroxidase to a cation radical (Barr and Aust, 1993).

5.4 Dissolved Oxygen Production from Conjugated HRP-E.coli Antibody

The dissolved oxygen production was tested with the use of varying concentrations of conjugated HRP- *E. coli* antibody and the substrate with enhancer ABTS. The commercially available conjugated antibody had a starting concentration of 1.288μM HRP. This antibody was added in a solution with distilled water, buffer, and ABTS. The reaction was started by the introduction of hydrogen peroxide into the beaker. Time zero represented the dissolved oxygen concentration with no hydrogen peroxide in system. For the commercial conjugated antibody, the dissolved oxygen production is shown in Figure 5.5a. The commercial conjugated antibody did offer a slight increase in dissolved oxygen. This increase was similar to the graphs of HRP without the use of ABTS. However, since the starting molarity of the conjugate was 1.288μM, in order to achieve testing at a higher molarity of HRP, very high volumes of the conjugated would need to be used. For instance, testing at 6 X 10^{-8}M HRP would

only allow for three uses of the commercially available product (1 milliliter volume). This would be very costly and limiting for this project and not satisfy the ten uses per vial of commercial conjugated antibody. The use of a higher molarity conjugate would allow testing to be achieved at higher concentrations HRP with the use of smaller quantities of conjugated antibody, offering the signal increase and dissolved oxygen production similar to those achieved with HRP enzyme free in solution.

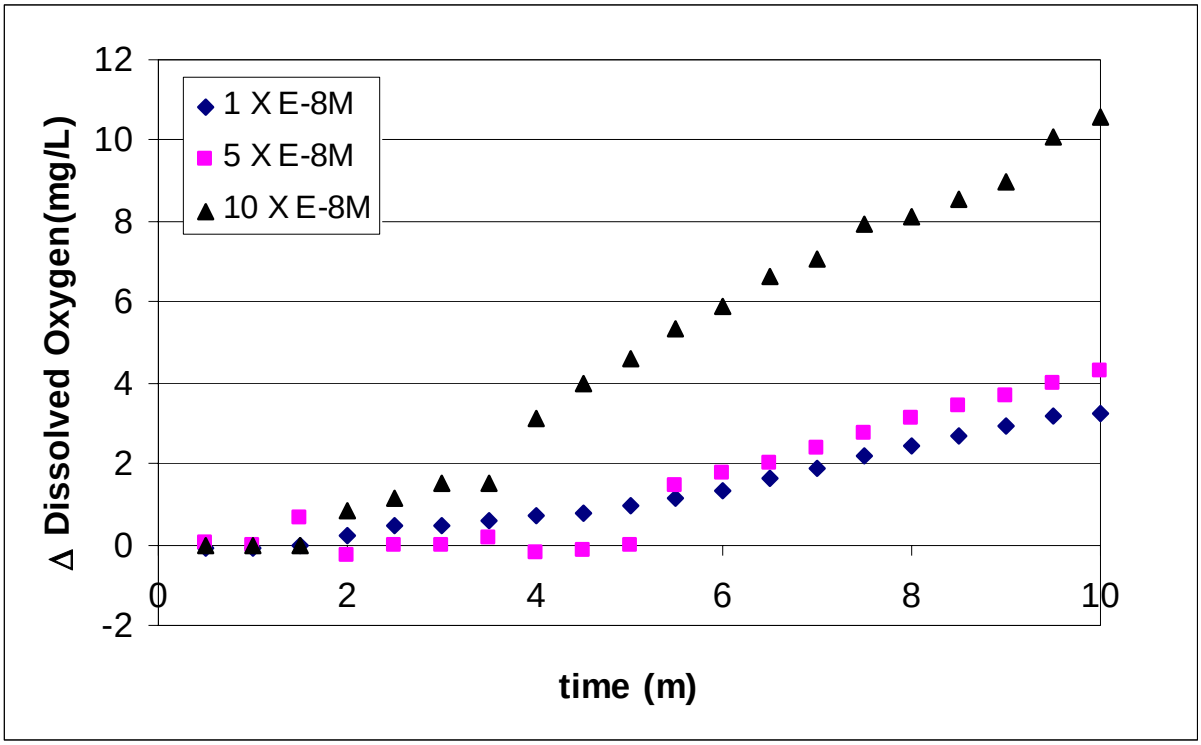

Figure 5.4: Dissolved Oxygen Production with the use of HRP-C, Hydrogen Peroxide, and ABTS

A custom conjugated HRP-*E. coli* antibody was made using the Sure Fire Conjugation Kit (KPL) and purchased antibody. The instructions for the conjugation method are found in the Appendix B. The same experiments conducted with the commercial antibody were repeated with the custom conjugated antibody. Since the

custom conjugated had an initial molarity of 30μM HRP, very little volume of antibody conjugate was used in comparison with that of the commercial product.

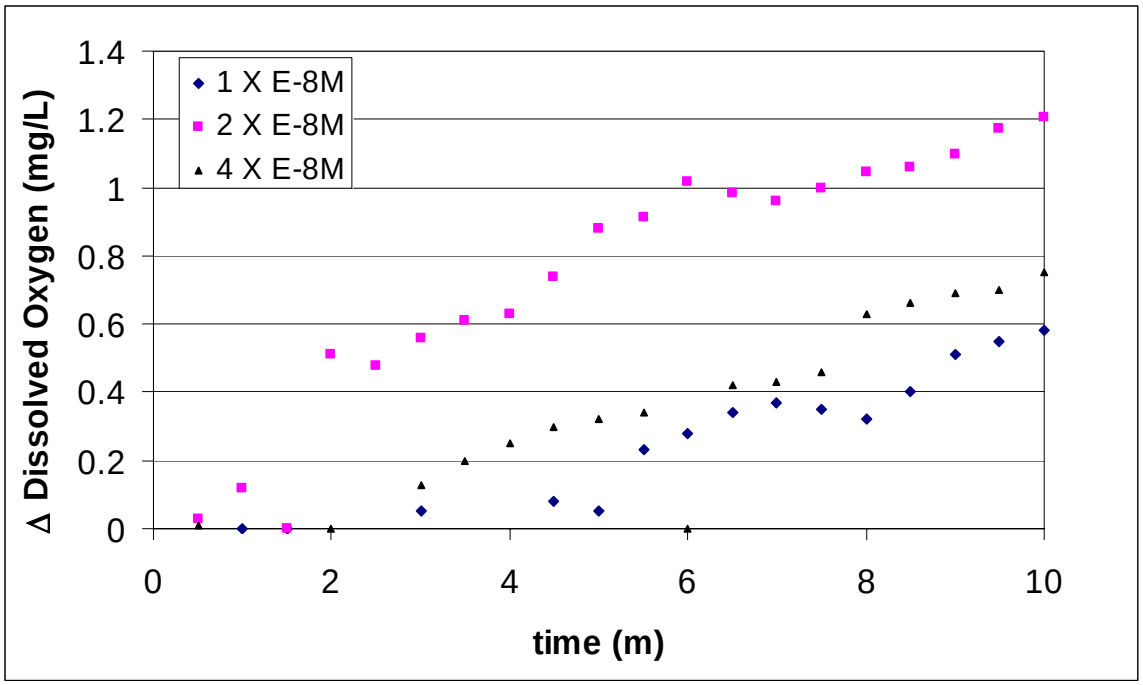

Figure 5.5a: Dissolved Oxygen Production from Commercially Conjugated HRP-*E. coli* Antibody with Hydrogen Peroxide and ABTS

Therefore, testing with a higher molarity HRP- conjugated antibody (greater than 4 X 10^{-8}M) was possible. Higher HRP concentrations increased dissolved oxygen concentration greater than 3mg/L in ten minutes. The average dissolved oxygen increases in the three trials at is shown in Figure 5.5b. The same trend is seen with these tests as seen with other HRP testing. In general, increasing HRP concentration with a constant substrate concentration, namely hydrogen peroxide with enhancer ABTS, created a higher concentration of product (dissolved oxygen) over time. The making of a custom conjugated antibody allowed repetitive experiments to be conducted by minimizing the volume of conjugate used for testing. The commercial conjugated antibody had a molar ratio of HRP: antibody of 4:1, whereas the custom conjugated antibody was able to achieve a ratio of up to 25:1 with suggested ratio of 10:1. Therefore, the custom

conjugated antibody would be utilized in testing of the amperometric biosensor for *E. coli* O157:H7 experiments.

Figure 5.6 shows a comparison of the HRP enzyme and conjugated HRP (both custom and commercial). The concentration of 1 X 10^{-8}M HRP is graphed in Figure 5.6. From this graph, after 10 minutes, there is more than 3 mg/L dissolved oxygen difference between the free HRP enzyme and conjugated HRP. This difference can be interpreted by evaluating the reduction in enzyme activity occurring during the conjugation process.

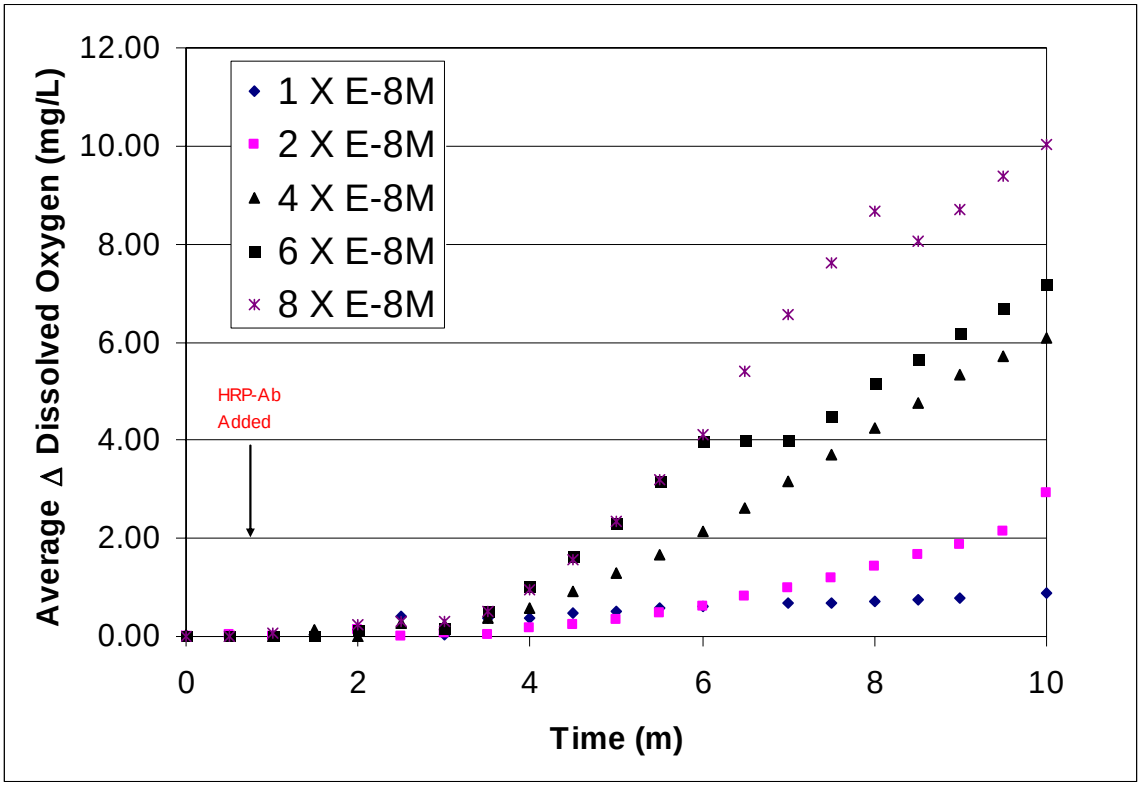

Figure 5.5b: Dissolved Oxygen Production from Custom Conjugated HRP-*E. coli* Antibody with Hydrogen Peroxide and ABTS

The initial velocity at 6 minutes can be used to compare the reduction in enzyme activity. At 6 minutes, the free enzyme HRP had a Vo value of 61.88µM/minute, while the commercial antibody had a Vo value of 8.75µM/minute, and custom made antibody's Vo value was 18.75µM/minute. The commercial conjugate had a reduction in enzyme

activity (from the HRP enzyme in solution) of approximately 86%. The custom conjugate had a reduction of enzyme activity of 70% from the HRP enzyme of the same molarity. Since the commercial conjugate and custom conjugate had similar enzyme activity, either would be able to be used for this process. However, the due to the higher ratio of HRP: antibody in the custom conjugate, smaller quantities of expensive immunochemicals are needed for analysis.

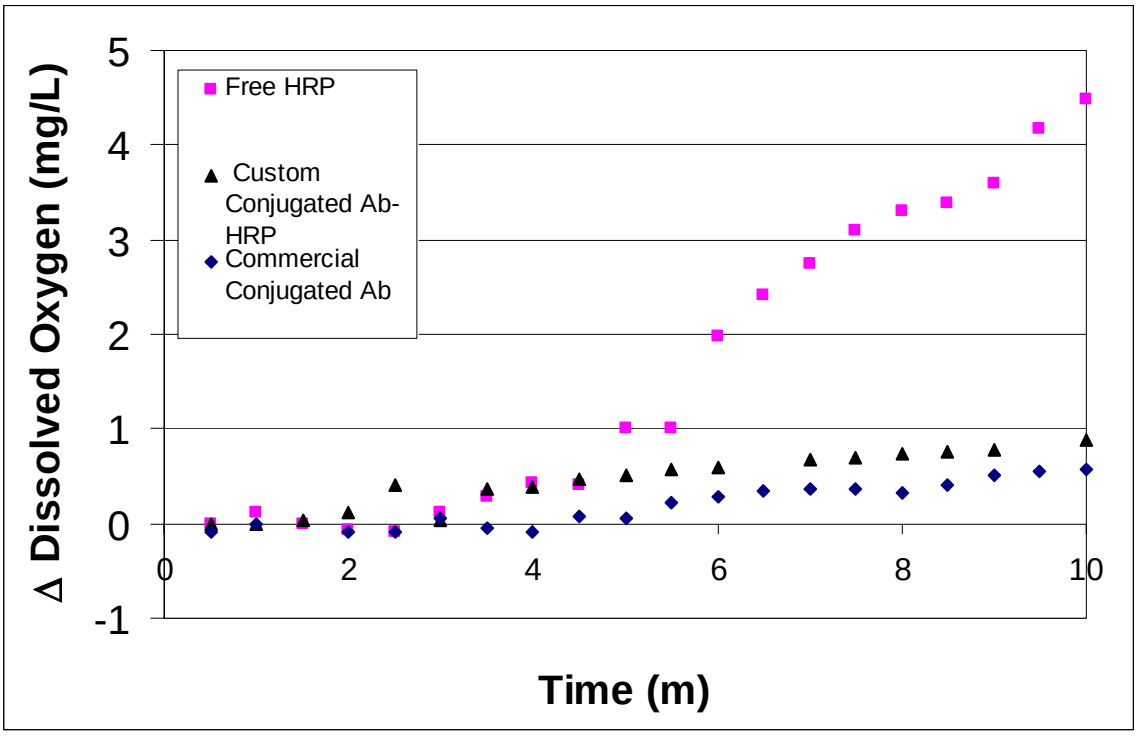

Figure 5.6: Comparison of Unconjugated and Custom Conjugated and Commercial Conjugated HRP-Ab at 1 X 10^{-8}M HRP

5.5 Temperature

Temperature effect on dissolved oxygen production was evaluated for the amperometric biosensor system. It is difficult to characterize all lake temperatures. For this

application, the target area is Lake Pontchartrain located in southern Louisiana, where the range of water temperatures is usually from 10-30°C (U.S Geological Survey, 2002). However, temperatures can even vary within an area due to unforeseen circumstances. It was the intent of this research to test the average temperatures from an extreme low to an extreme high. Therefore 4-40°C was chosen as the range of temperatures to test the biosensor. Figure 5.7 shows the results from the temperature experiment. Since the biosensor showed successful oxygen production within these temperatures commonly recorded in Lake Pontchartrain, we can conclude that temperature would not be the limiting factor in amperometric biosensor's ability to monitor *E. coli* O157:H7 concentration. Also, dissolved oxygen concentration using a Clark electrode is a function of temperature (temperature is directly proportional to dissolved oxygen concentration). Therefore, a need to calibrate the change in dissolved oxygen concentration at varying temperature is necessary for the amperometric biosensor.

5.6 pH

The pH range at which the amperometric biosensor would be most effective was evaluated. This is labeled as the optimum pH of the amperometric biosensor. This pH profile is found in Figure 5.8. In this experiment, the probe with outer insert was submerged in the substrate consisting of hydrogen peroxide, ABTS, and buffer at a given pH as outlined in the methodology. The dissolved oxygen profile was taken over a ten minute period.

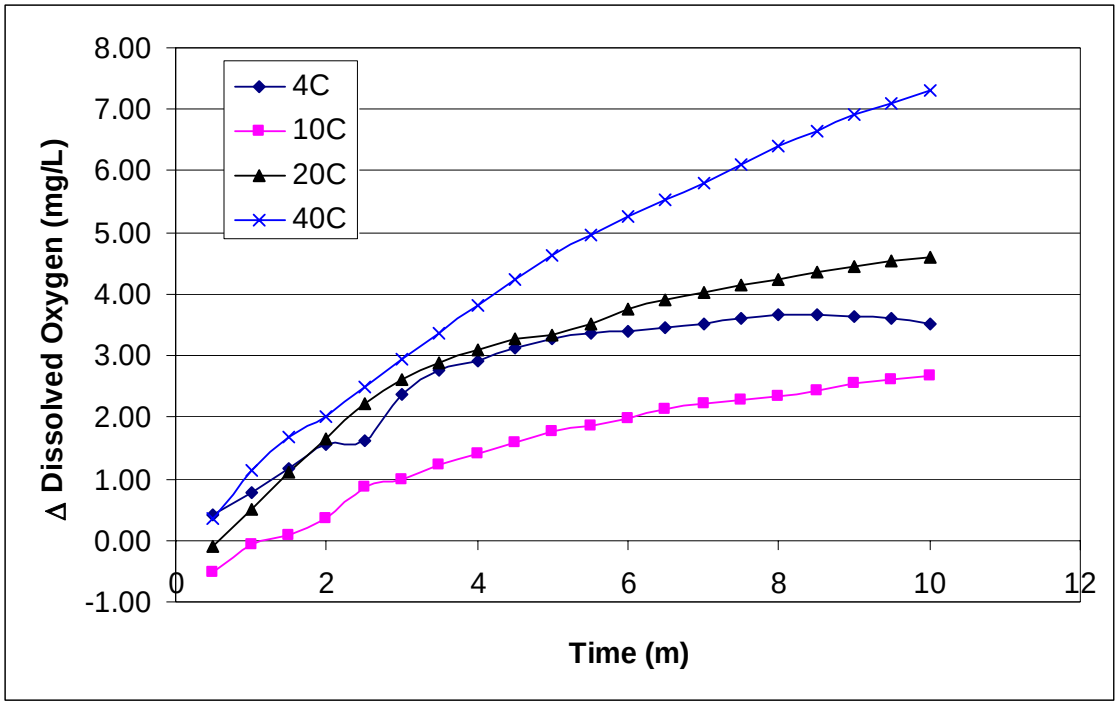

Figure 5.7: Dissolved Oxygen over Time at Varying Temperatures

The maximum rate of oxygen production occurs at pH 6.7-7.6. For pH values under 6.7, instead of oxygen production, the sensor seems to experience a reduction in oxygen. This can be noted by the inverse hyperbolic curve in which the dissolved oxygen concentration decreases over time. At pH 6.7 and 7.6, the dissolved oxygen concentration increases over time. Hence, the acid media would be unfavorable for the catalase activity. This agrees with the findings of Hernandez-Ruiz et al (2001) in which HRP-C in solution was tested. Hernandez-Ruiz et al (2001) reported that the oxygen production plateaus over pH6.5-8.5. However, *E. coli* O157:H7 is known to be able to survive in acidic environments. The amperometric biosensor is projected to be used in monitoring stations found at sites along a lake, for example Lake Pontchartrain. Lakes naturally maintain pH levels between 6.5-8.5, which agreed with the optimum operable pH range of the amperometric biosensor. If the sensor were to be used in detection of a

more acidic environment, for instance, *E. coli* O157:H7 detection in apple juice, there would be a great need for a pH adjustment in final substrate solution to reduce the affects from lowered sample pH.

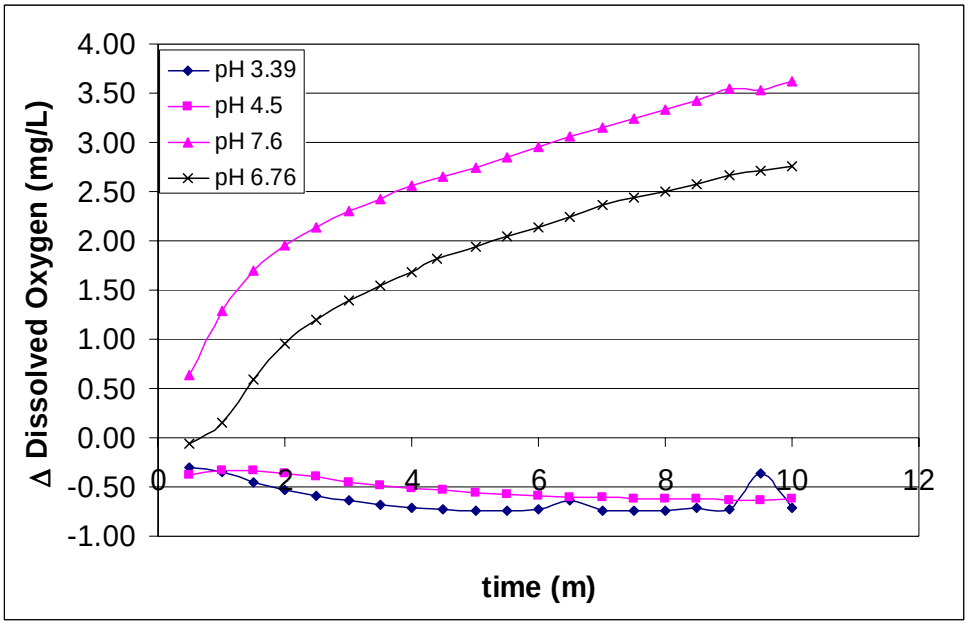

Figure 5.8: Change of Dissolved Oxygen versus Time at Various pH

5.7 Bacterial Concentration Curve- Using Amperometric Sensing

From the initial phase bacterial biosensor tests, some major changes occurred in the sensing procedure. The first change is the use of separate solutions for bacteria, washing, and final detection (substrate). These changes mimic how the biosensor could be used in the future for detecting field samples. Exposing the biosensor to bacteria is analogous to submersion into a water sample, which could have bacterial cells present, namely *E. coli* O157:H7. The washing step that was incorporated played a very important role in bacterial sensing. This step allowed unbound HRP and unbound bacterial cells to be washed from the membrane. This would help insure that what was bound to the membrane, which would go into the final substrate, would be specific to detecting *E. coli* O157:H7. This included attached conjugated HRP-*E. coli* antibody and *E. coli*

O157:H7, if present in solution. The last step would be the final process in sensing which included submerging the biosensor into a substrate, and subsequent oxygen production.

Figures 5.9a-b display the results obtained when using the amperometric biosensor to detect *E. coli* O157:H7. The average change in dissolved oxygen concentration for each concentration of *E. coli O157:*H7 is shown in this figure. *E. coli* concentrations in the range 0-5000 cells/ml were tested in this study. The curves are hyperbolic in nature reaching steady state after about 6 minutes from the time the HRP enzyme starts reacting with hydrogen peroxide. From this figure, there is a distinction in concentration at steady state. The initial velocity (Vo) is recorded in the table below, Table 5.2. Figure 5.10 shows *E. coli* concentration versus initial velocity. Since initial velocity is determined when there is a constant increase in oxygen production, which is calculated before the system reached steady state, a time of 1 minute was used for these calculations. Since the calibration curve was not linear in this time frame (R^2 value was low (0.145), and the standard error was highest at this time frame) we can conclude that initial velocity can not be used to determine dissolved oxygen changes with changing bacterial concentrations. The change in dissolved oxygen concentration at steady state is recorded after 10 minutes in Figure 5.10a-b.

From Figures 5.9a and 5.11a, it can be interpreted that there is a difference in change in dissolved oxygen (increase) at steady state at for varying *E .coli* concentrations (cells/ml) in solution. For instance, the average change in dissolved oxygen for 0 cells/ml present is 6.2mg/ml. After evaluating the average change in dissolved oxygen in Figure 5.9a-b and comparing that with Figure 5.11a, it can be assumed that after 10 minutes a

change in dissolved oxygen over 6.2 ±1.25mg/ml (α =0.05) represented no cells present in solution.

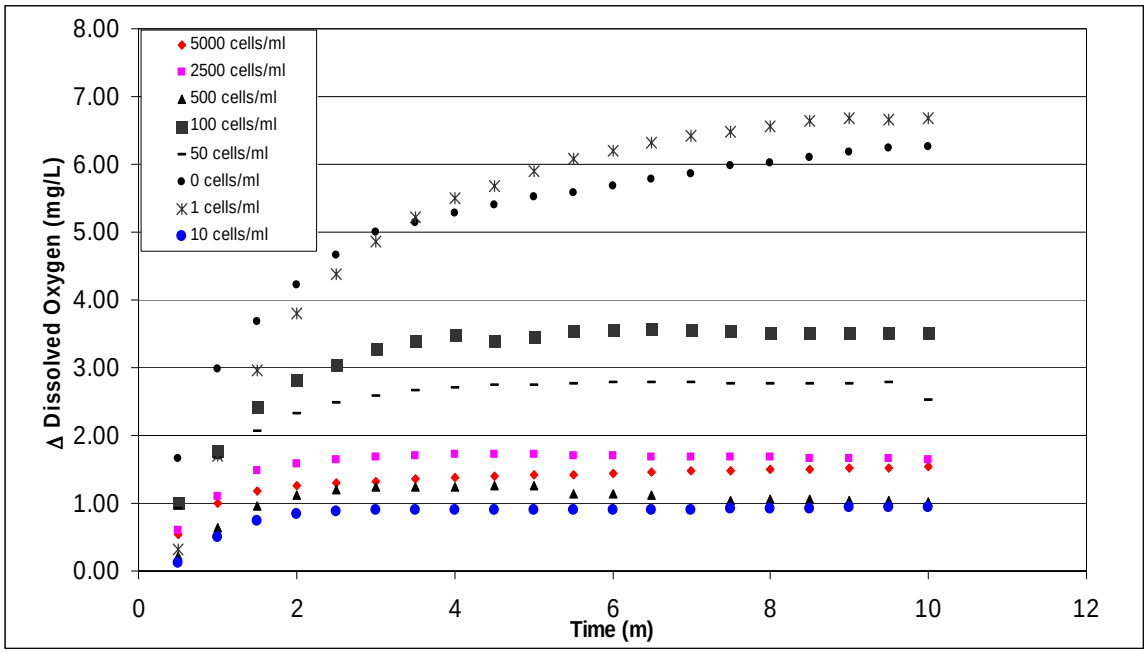

Figure 5.9a: Average Dissolved Oxygen Production at Varying Concentrations of *E. coli* O157:H7

This is consistent throughout the study. For 50 cells/ml and 100 cells/ml, the average change in dissolved oxygen at steady state is 2.52 ± 0.73mg/L (α =0.05) and 3.53± 0.50mg/L (α =0.05), respectively. From this information, it can be applied that below 4mg/ml change (increase) in dissolved oxygen concentration indicated that at least 10 cells/ml of *E. coli* O157:H7 are present in system. For concentrations of 500 cells/ml and above there is a great degree of standard error (0.61-1.02). The standard deviation for these values range from 1.06-1.7. However, the bacterial concentrations equal to and

greater than 500 cells/ml do not have increases in dissolved oxygen over 4mg/L with consideration of high standard deviations, as seen with all bacterial solutions containing bacteria. Hence, this is consistent with the notion that increases in dissolved oxygen below 4mg/ml represent at least 10 cells/ml *E. coli* O157:H7 present.

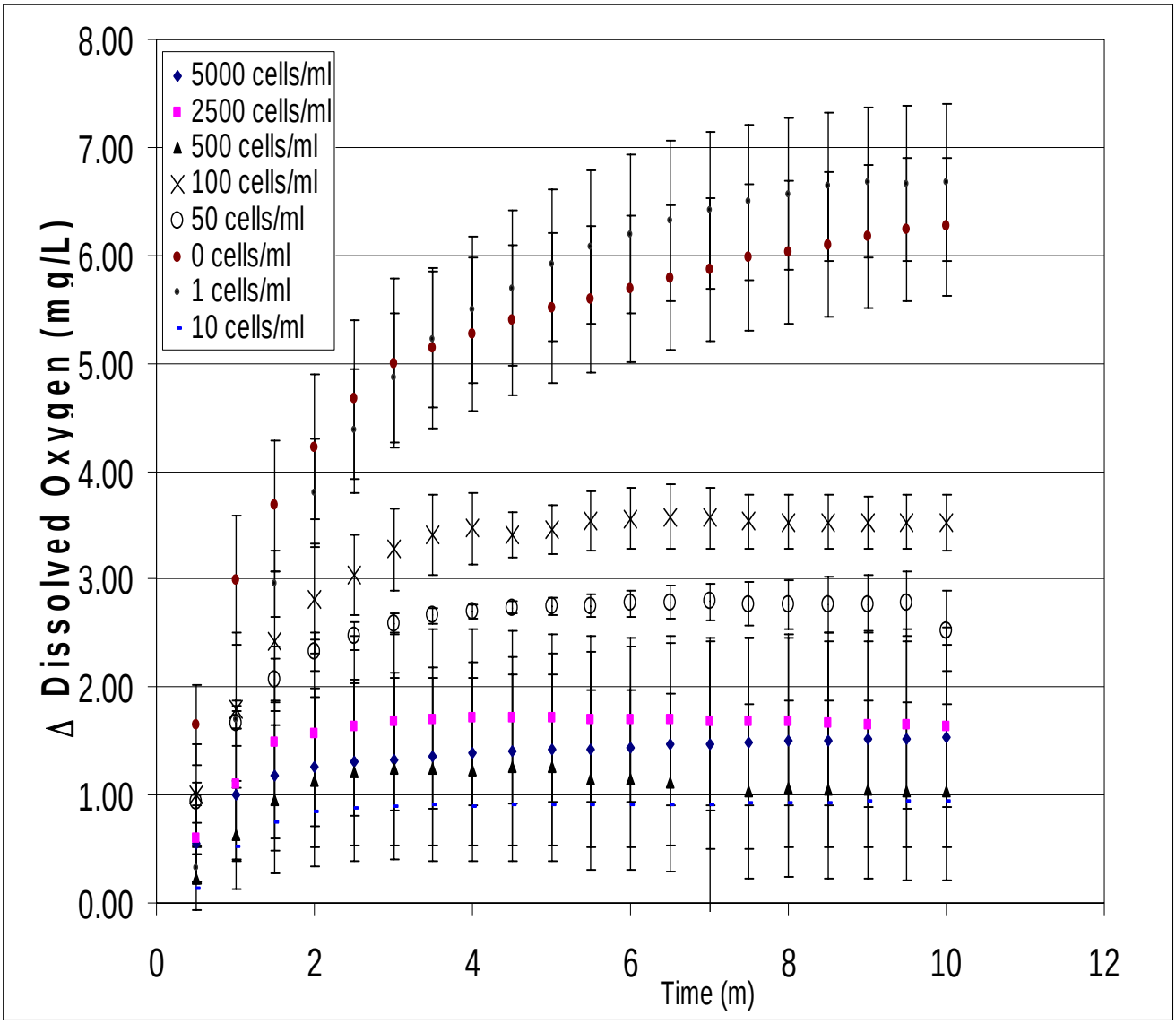

Figure 5.9b: Average Dissolved Oxygen Production at Varying Concentrations of *E. coli* O157:H7 with Standard Error

Table: 5.2: Initial Velocity for Hydrogen Peroxide and ABTS with HRP after 1 minute

E.coli O157:H7 (cells/ml)	Vo (μM/minute)
5000	31.56
2500	34.38
500	19.69
100	55.94
50	52.19
0	93.13

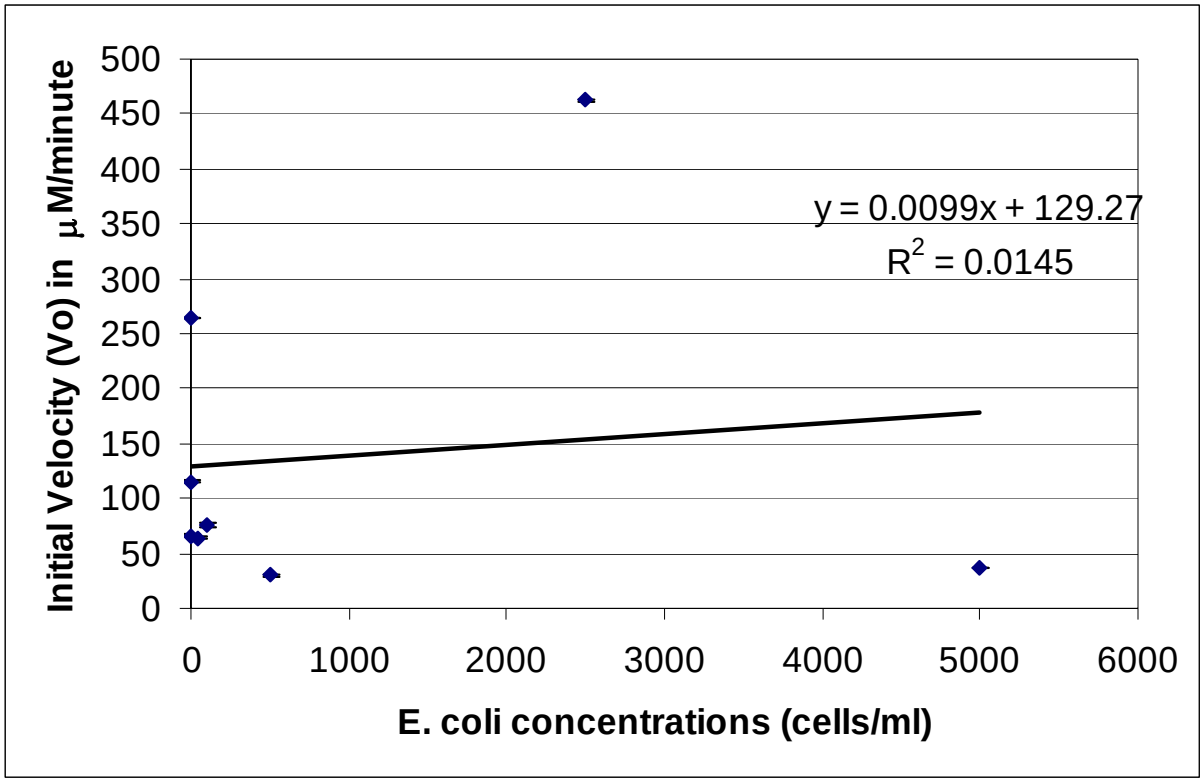

Figure 5.10: Initial Velocity versus *E. coli* concentration

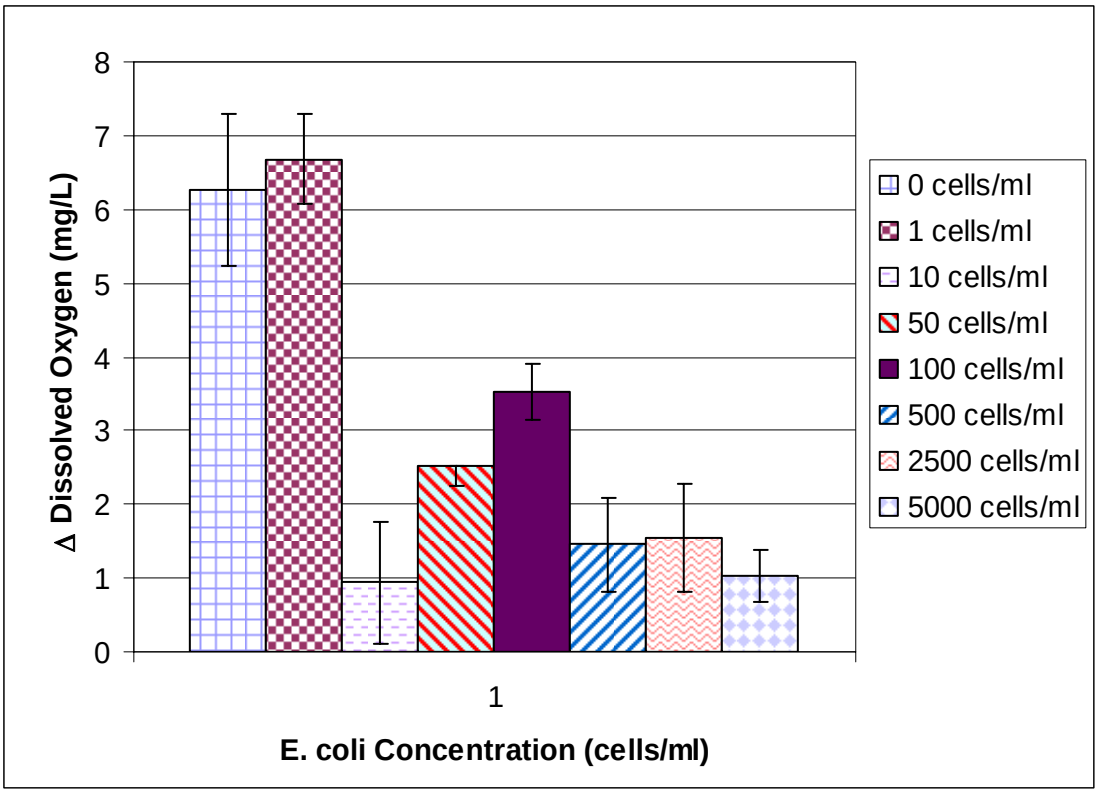

Figure 5.11a: Average Dissolved Oxygen Production at Varying Concentrations of *E. coli* O157:H7 at 10 minutes steady state

The concept that an amperometric biosensor can detect *E. coli* O157:H7 is one that can be accepted with many limitations. From this study, the amperometric biosensor does offer a way to determine if *E. coli* O157:H7 cells are present. However, the ability to distinguish between bacterial concentrations does offer a challenge with the use of one conjugated antibody. This is evident from figure 5.11b, the plot of *E. coli* concentration versus change in dissolved oxygen, where the R^2 value is 0.5936. From the low R^2 value of the linear curve, it was concluded that the amperometric biosensor does not offer the ability to quantify bacterial (*E. coli* O157:H7) concentrations. The dissolved oxygen readings also varied from one testing period to another at the 0 cell/ml concentration. This variation could have been due to differences in enzyme activity, changes in

dissolved oxygen from the water supply, or membrane loading. There may be a need to calibrate the amperometric biosensor at the beginning of a new set of readings. This would minimize the variations in the sensor and reduce the need to estimate what changes might occur due to unforeseen circumstances.

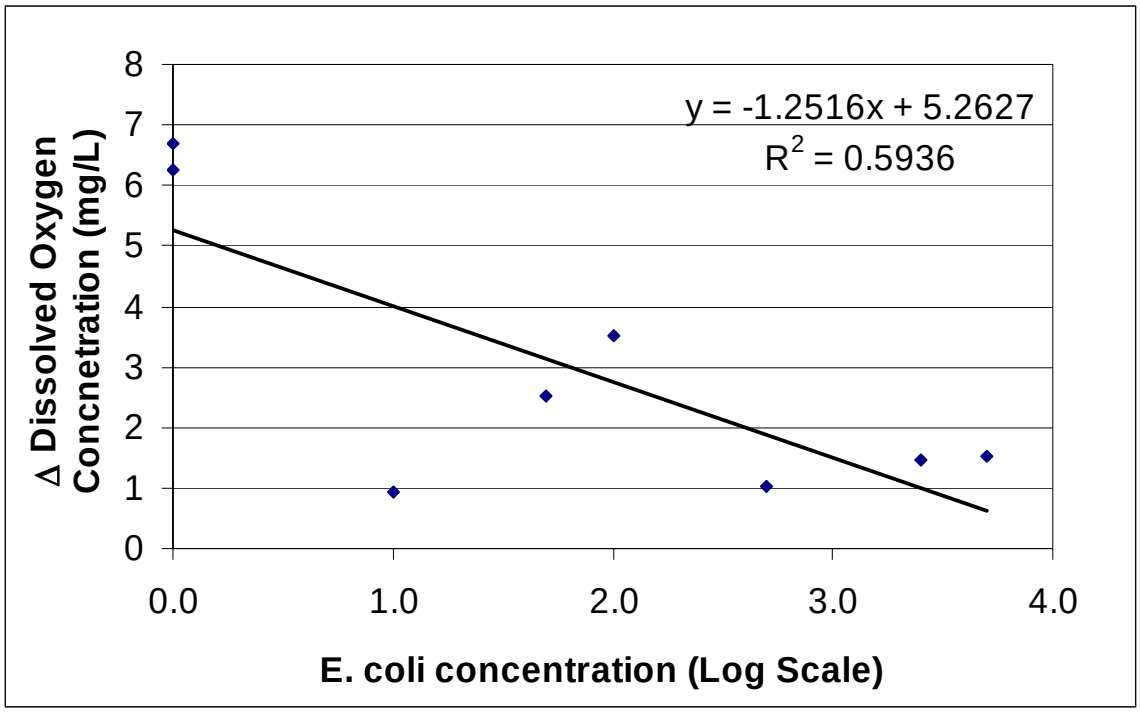

Figure 5.11b: Average Dissolved Oxygen Production at Varying Concentrations of *E. coli* O157:H7 at 10 minutes steady state

Although it is unclear to exactly which amine groups HRP is conjugated to, this may have contributed to the decrease in product with the attachment of cells. If the amine groups are located within the *E. coli* binding site, the binding of *E. coli* could "block" the substrate from binding with the enzyme. On the other hand, if none of the HRP enzyme is conjugated to the binding site, the large size of the *E. coli* O157:H7 cell

could physically block the substrate from binding with HRP. Characterization of the behavior of blocking can not be concluded in the study.

5.8 Alternate Substrate

The results found using the substrate TMB instead of hydrogen peroxide and ABTS are shown in Figure 5.12. TMB is an alternate substrate that has an apparent colorimetric change when reacting with HRP. Therefore if the concept of blocking was a phenomena this system was experiencing, then the same pattern should be seen with TMB as seen with ABTS and hydrogen peroxide. From comparing figures 5.11b and 5.12, the graphs have similar trends, which is a decreasing slope. The solution with no cells present has the highest absorbance value, therefore creating the most product from the interaction of enzyme and substrate. The 50 cells/ml concentrations had a lowered showed a lowered absorbance, while 100 cells/ml was slightly higher absorbance value than 50 cells/ml. A similar pattern was noticed with the dissolved oxygen experiments. The reason in which this variation was seen between concentrations could not be determined in this study. The 5000 cells/ml (highest concentration tested) clearly had the least absorbance. The distinct response was not evident with the dissolved oxygen experiments. The plot of absorbance versus *E. coli* concentration is linear between 0-5000 cells/ml. In summary, sensing with the use of a single conjugated antibody showed better quantification of bacterial concentration when colorimetric change utilizing the TMB reaction was evaluated instead of the use of change in dissolved oxygen. The TMB reaction supported the hypothesis that the binding of antigen may block the HRP that was conjugated to the antibody, therefore reducing the interaction of the enzyme and

substrate, and product formation (colorimetric for TMB). This phenomenon is represented with the negative slope in Figure5.12.

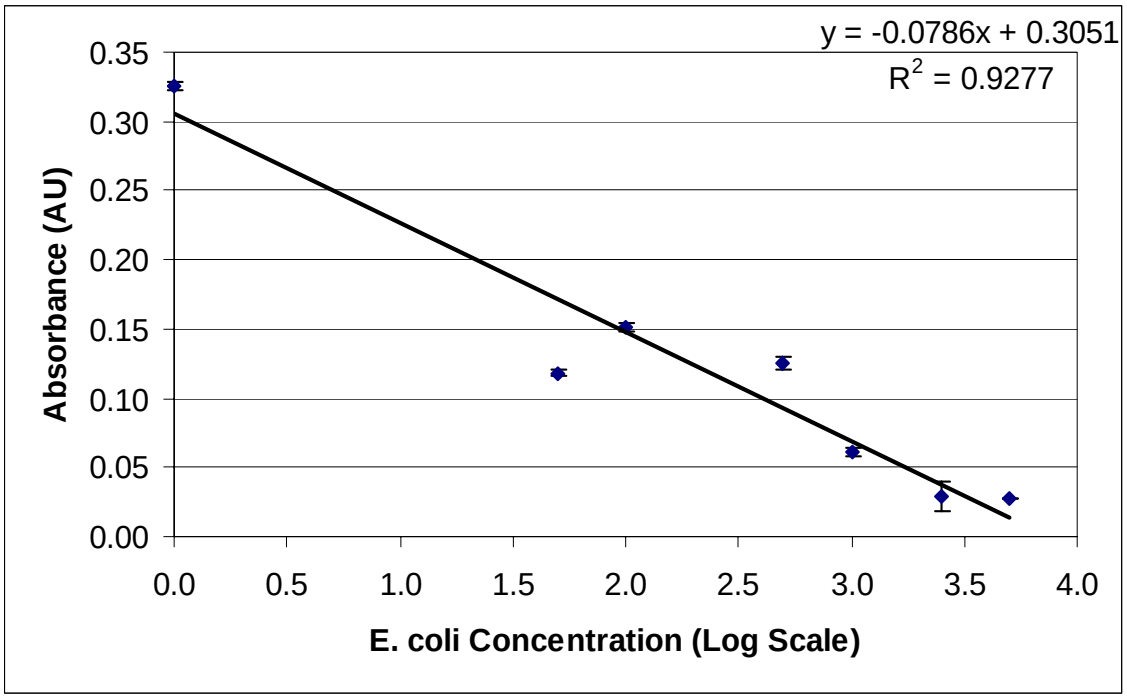

Figure 5.12: Absorbance versus Bacterial Concentration

5.9 *E. coli* O157:H7 Testing with the Use of Two Antibodies

The amperometric biosensor was evaluated with the use of a sandwich antibody assay. The outer membrane was prepared with the unlabeled *E. coli* O157:H7 antibody which was utilized to make the HRP conjugated antibody. Next, the amperometric biosensor was exposed to the bacterial solution. The membrane with unlabeled *E. coli* antibody was washed in 0.1M tris solution. Then, the conjugated HRP-*E. coli* was applied to the outer insert. The membrane was again washed with 0.1M tris solution. Finally, the amperometric biosensor was exposed to the substrate and measurements were taken and analyzed. Details regarding this procedure can be found in the Methodology section.

The response of the amperometric biosensor with sandwich assay to varying concentrations of *E . coli* O157:H7 is presented in Figure 5.13. The biosensor signal was linear from 0-100 cells/ml and therefore this region is shown in Figure 5.13. Beyond 100 cells/ml the change in dissolved oxygen concentration was non-linear and could not be distinguished from 100 cell/ml. The amperometric biosensor with the use of two antibodies (sandwich) assay provided a better means of quantification than with the use of one conjugated antibody. The sandwich colorimetric reaction with the use of a substrate like TMB offered better sensitivity than the amperometric biosensor. This may have been due to the high sensitivity of TMB to the varying HRP concentrations. The sandwich assay with the amperometric biosensor tripled testing time as compared with the one conjugated antibody. A minimum of one hour was needed for detection of bacteria which included many washing steps. Hence, the amperometric biosensor with sandwich assay, although needed longer testing time, offered a way for bacterial quantification.

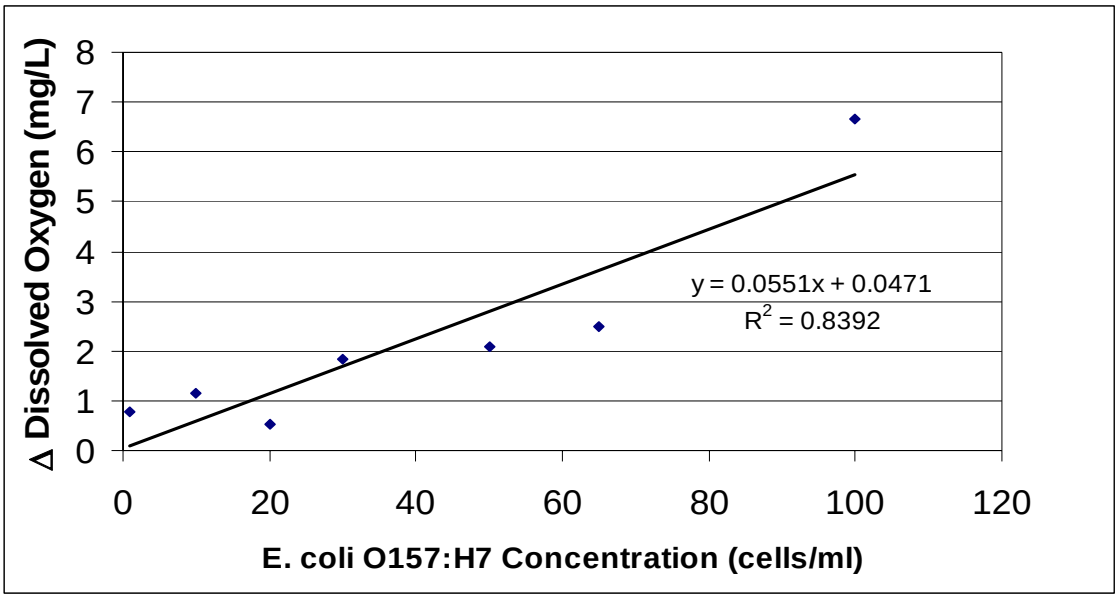

Figure 5.13: Change in Dissolved Oxygen Concentration versus Bacterial Concentration for amperometric biosensor with the use of two antibodies

5.10 Conclusions

It was necessary to find appropriate substrate and enzyme concentration for the amperometric biosensor system. The effects of environmental factors, pH and temperature, on the amperometric biosensor's performance were evaluated to determine if this would parallel those environmental factors commonly seen at monitoring stations used to monitor water quality. The use of a custom conjugated antibody offered many benefits to the amperometric biosensor, such as ability to control the molar ratio of HRP: antibody and minimize the volume of antibody used. The concentration of 6×10^{-8}M HRP was the minimum concentration needed to generate a distinguishable change in dissolved oxygen when attached to a nitrocellulose membrane and conjugated to *E. coli* antibody. These factors were taken into consideration when using the amperometric biosensor to detect heat sterilized *E. coli* O157:H7 cells. The final testing of bacterial cells consisted of a three step process: exposing of sensor into water sample, wash, and exposing to a substrate. This process lasted a total of 17 minutes, with 10 minutes for signal (dissolved oxygen change) generation. Hence, a water sample from Lake Pontchartrain may require additional time for preparation. The final results offered a indication of bacterial cells. However, it was difficult to distinguish between bacterial concentrations without the help of a second antibody. Quantification of the organism with the TMB reaction and use of a single conjugated antibody was proved viable. The absorbance versus bacterial concentration had a negative slope, which may signify that some blocking or reduction in enzyme activity occurs with the binding of the antigen. Overall, the results show that an amperometric biosensor can be used to indicate the presence of bacterial cells and therefore help identify contamination. Conversely,

quantification of bacterial concentrations was not possible with the single conjugated antibody and substrate system. The detection limit for this system was 10 cells/ml when as little as 1 cell/ml were tested with no significant response compared to the absence of *E. coli* cells.

5.11 Comparison with Other Rapid Detection Methods

The amperometric biosensor can be compared with other methods to detect *E. coli* O157:H7. The detection time is for the sensor was about 17 minutes for heat sterilized pure culture *E. coli* O157:H7 cells. This is compatible with microarrays, fiber optic, and integrated systems which had a detection time of less than one hour. The detection time is less than most other rapid detection systems including ELISA, RT-PCR, and laser induced fluorescence. However, the amperometric biosensor was not able to quantify the organism. This is a major difference in this system and other rapid detection mechanisms. In addition, the ELISA system of detection is more sensitive than the amperometric biosensor. For instance 3μL of a 1 μg/ml antibody-HRP conjugate is sufficient to create a measurable absorbance reading utilizing the ELISA system. The amperometric biosensor required at least 1000 times as much conjugated antibody to create a signal which was not distinguishable between concentrations. However, the instrumentation (Clark electrode) required for the amperometric biosensor is one that is readily available at monitoring stations located at Lake Pontchartrain and could easily be incorporated into an monitoring station. Furthermore, the amperometric biosensor with a single, conjugated antibody only required two washing steps and the adding of one substrate to a prepared solution. This is significantly lower than ELISA and most other biosensors which require a great number of washing steps and substrates for the reaction. These

systems usually require the use of two antibodies, while the amperometric biosensor requires only one antibody. ELISA, molecular beacons, PCR, and other rapid detection methods usually require some degree of expertise and complexity. The amperometric biosensor utilizes an easy to use probe that requires no level of expertise to operate. The amperometric biosensor with one conjugated antibody offered many advantages to the current rapid detections methods.

CHAPTER 6. OVERALL CONCLUSIONS AND FUTURE CONSIDERATIONS

It was hypothesized that the binding of bacteria to the conjugated antibody would decrease the dissolved oxygen production. As discussed earlier in Chapter 2-Review of Literature, the reaction that takes place between the substrate hydrogen peroxide and the enzyme horseradish peroxidase (denoted by HP or HRP) is as follows:

$$H_2O_2 \xrightarrow{HP} O_2 + 2H$$

HP = Horseradish peroxidase

In the equation above, the binding of the substrate hydrogen peroxide and enzyme, horseradish peroxidase, would cause an increase in oxygen production. The reaction would take place in the final phase of testing with the help of ABTS. In this study, the horseradish peroxidase was attached to *E. coli* O157:H7 at various amine groups throughout the antibody. It is unclear as to exactly where these amine groups are located on the antibody. However, if some of the amine group attachment sites where located in the *E. coli* O157:H7 binding region, or Fab region, then the binding of bacteria could "block" some of the conjugated HRP from reacting with the substrate, causing lowered oxygen production. On the other hand, if a solution contained no bacterial cells, HRP would be free to bind with hydrogen peroxide, creating a maximum production in oxygen. This phenomena is studied in Xu and Sulieman's (1997) reusable amperometric biosensor to detect cortisol where luminescent testing showed a reduction in HRP activity with the binding of the antigen.

The amperometric biosensor technology may be used for detecting bacterial cells, mainly *E. coli* O 157:H7. Through the use of a recordable change in dissolved oxygen concentration, the bench scale model of an amperometric biosensor was successful in detecting heat –sterilized *E. coli* O 157:H7 cells.

There was a great need to optimize and test the system's performance with varying parameters such as substrate concentration, enzyme concentration, pH, and temperature. Testing these parameters allowed a workable range in which the sensor can be used. Individual tests for bacterial concentrations were conducted in 17 minutes. Ten minutes was needed in order to test the signal generated, while 7 minutes was necessary for preparation (exposing into sample and washing).

Since the amperometric biosensor offered challenges in differentiating between concentrations in *E. coli* cells, the use of a second, unlabeled *E. coli* O157:H7 antibody was evaluated. After testing the amperometric biosensor, the results showed the second antibody improved the ability of the sensor to quantify bacterial concentrations.

The amperometric biosensor may be used to detect organisms other than *E. coli* O157:H7. There is no reason to believe that this technology is specific to the organism *E. coli* O157:H7. Antibodies which are specific to other fecal coliform bacteria can be utilized with the amperometric biosensor system. This may give more insight to fecal coliform contamination problems. There is also a need to test this system with different types of bacteria. This would allow a way to determine specificity for the target bacteria, *E. coli* O157:H7. This would also help to determine if there is a need for enrichment steps for water samples.

In the future, the bench scale amperometric biosensor may be automated and re-designed to be used at monitoring stations. One objective of this project was to design a bench scale system to test the linearity between dissolved oxygen and bacterial concentration with the use of one antibody. At the current stage, a three step process is necessary for detection, which may require water samples to be brought back to a lab. A portable design of the current system may be the next step for on-site applications.

A dual sensing technique may be applicable to the system. Although, not quantified in this study, there was an apparent colorimetric change in ABTS which is proportional to the change in dissolved oxygen concentration. The colorimetric change can be correlated with the change in dissolved oxygen; thereby, offering two ways to quantify the bacterial detection. If greater sensitivity is experienced with the ABTS colorimetric reaction as experienced with the TMB reaction, this may also allow more insight to the reduction in enzyme activity experienced when bacterial cells bind with the amperometric biosensor. The ABTS reaction utilizing colorimetric change and comparison of sensitivity to horseradish peroxidase should be explored.

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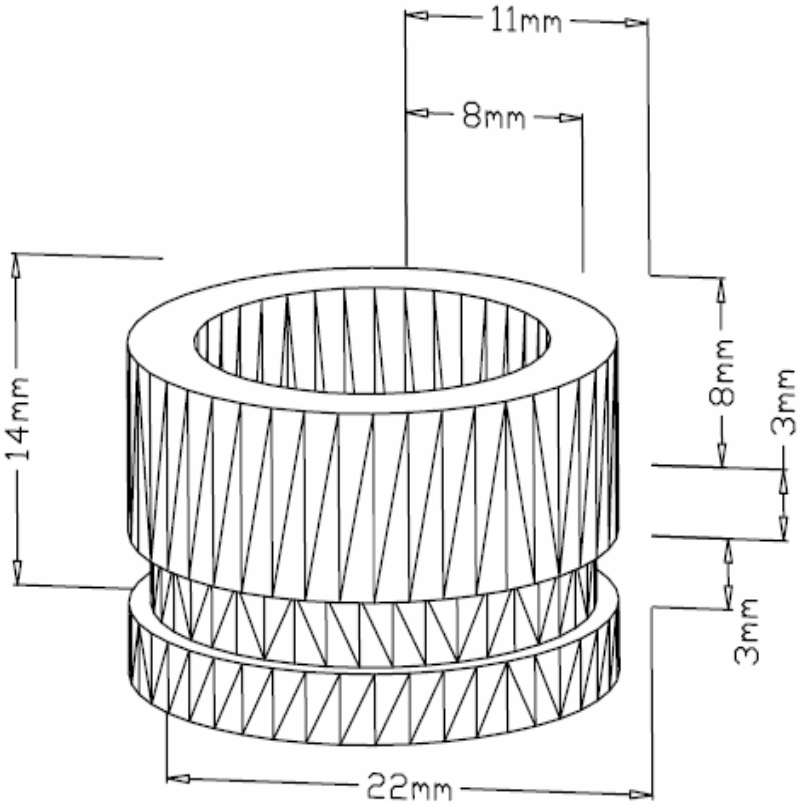
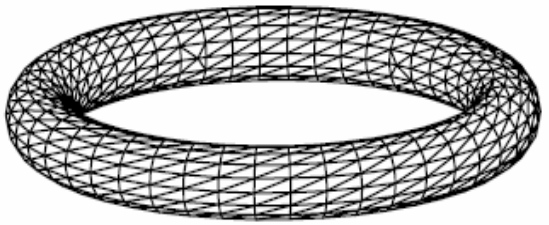
APPENDIX A
DRAWING OF OUTER INSERT

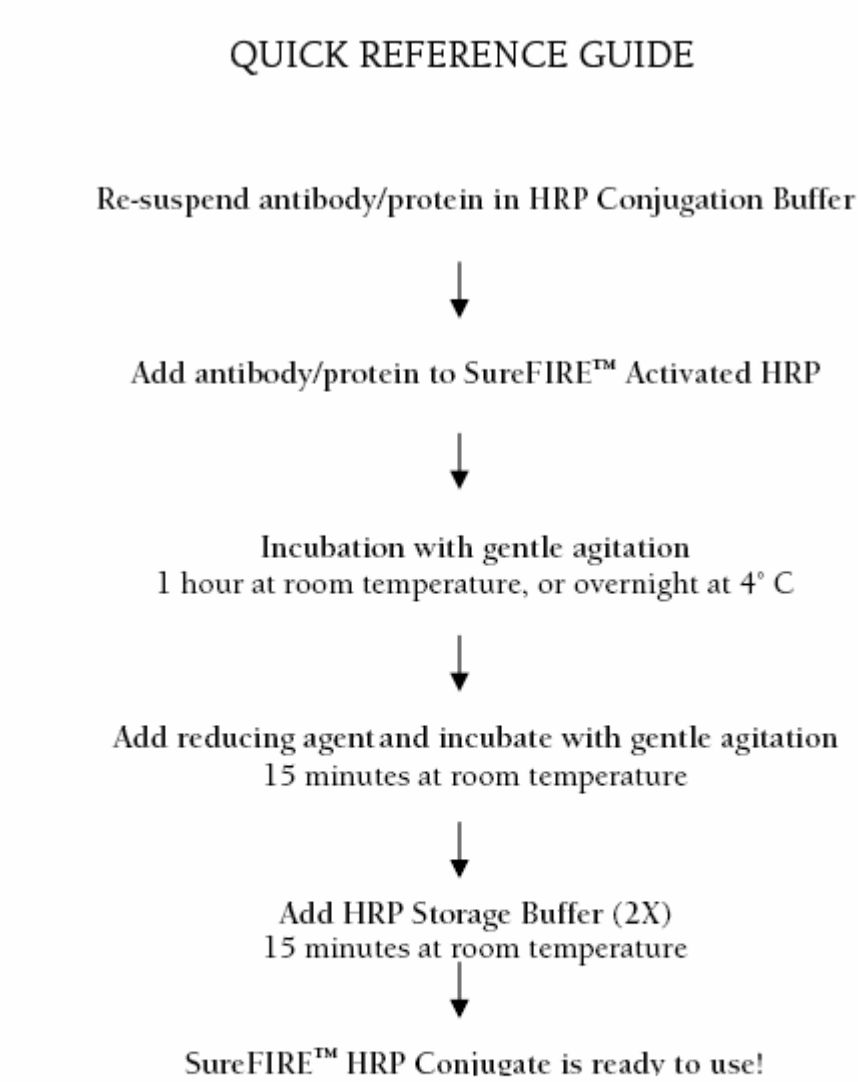




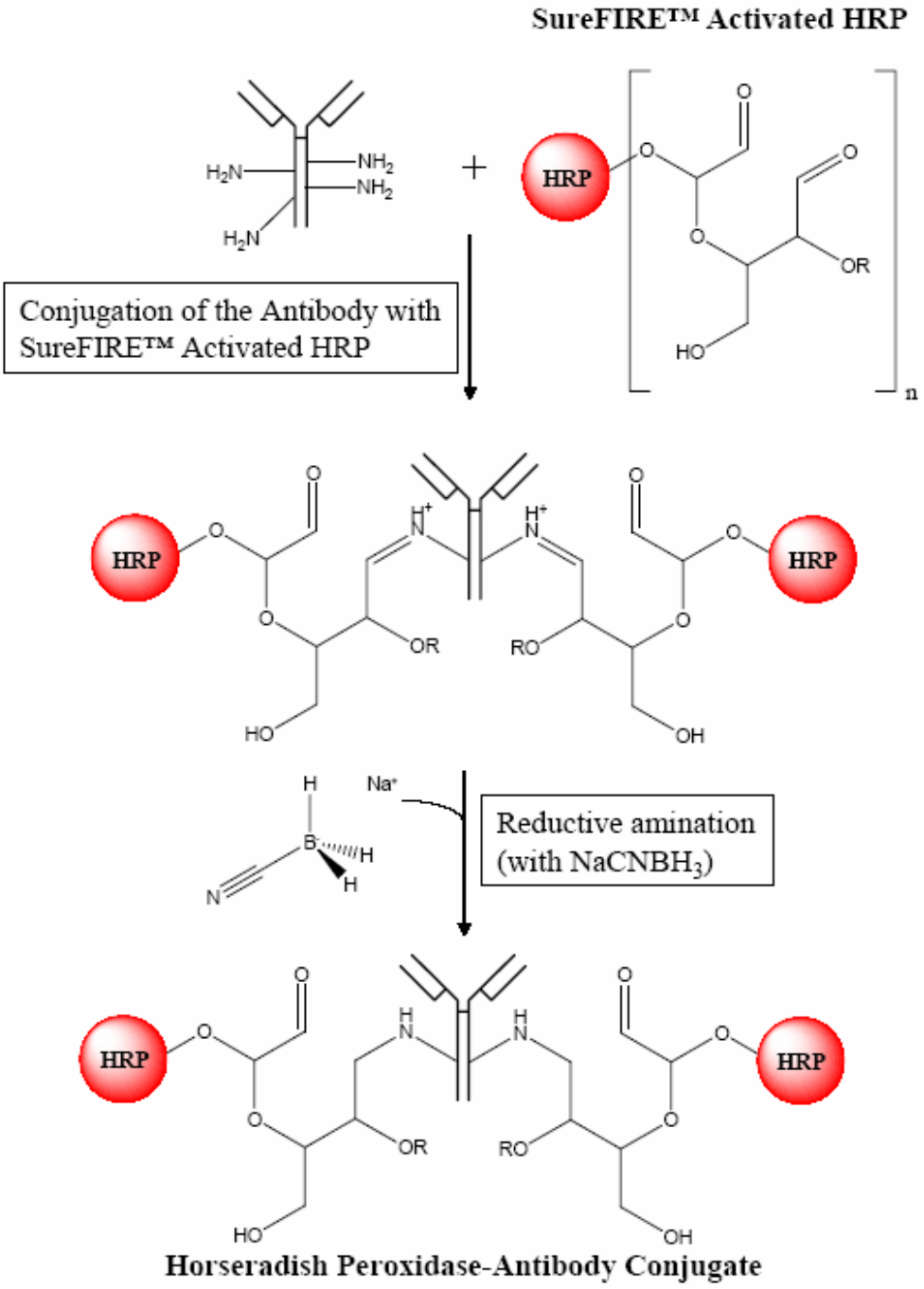
o-ring
1mm
23 r outer
18 r inner

APPENDIX B
CUSTOM CONJUGATION INSTRUCTIONS

Source:www.kpl.com



SureFIRE HRP CONJUGATION CHEMISTRY



PROCEDURE UTLIZED FOR PREPARING CUSTOM CONJUGATED ANTIBODY

All materials included in custom conjugation kit were used for preparation of conjugate. An unlabeled *E. coli* O157:H7 antibody was reconstituted at 1mg/ml utilizing HRP conjugation buffer found in kit. The antibody was allowed to remain in buffer for at least one hour. Next, one hundred microliters of the reconstituted antibody was added to a vial containing 0.3mg activated HRP and gentle agitation was applied for 20 seconds. This reaction was allowed to take place for one hour. Then 10 microliters of the reducing agent (found in kit) was added to the vial containing antibody and HRP. After fifteen minutes, the HRP storage buffer (found in kit) was added to the vial. The HRP storage buffer was allowed to remain in the vial for 15 minutes at room temperature before use of the final custom conjugated antibody. The custom conjugate was stored at 4°C for long term use. Each custom conjugated antibody was utilized within a week of preparation. The quantities used were calculated utilizing the tables below for a 10:1 molar ratio of HRP: antibody. The time to complete custom conjugation process was approximately 90 minutes.

Determine the required amount of SureFIRE Activated HRP for conjugation reaction:

Part 1: Determine the HRP:Antibody (or protein) molar ratio (see Table 1)

Section 1: Labeling immunoglobulin:
For antibodies with a molecular weight of ~150 kDa, the optimal amount of HRP required to conjugate with an antibody sample varies as a function of the amount of the antibody in the reaction. A molar ratio of HRP:Antibody of 10:1 is recommended for antibody samples of 0.1 mg or less. A molar ratio of 5:1 is optimum for antibody samples greater than 0.1 mg. Reducing the HRP:Antibody ratio (for example from 5:1 to 1:1, using less HRP) favors the production of lower molecular weight conjugates which may possess enhanced abilities to penetrate cell membranes.

For higher molecular weight antibodies (>700 kDa), a molar ratio of HRP:Antibody of 25:1 is recommended.

Section 2:Labeling other proteins and peptides:
For lower molecular weight proteins/peptides (MW <5 kDa), the optimal HRP:Protein ratio may be much lower (i.e. 1:1 to 1:10). Further titration of the amount protein/peptide may be required to determine the optimal condition for the specific protein sample.

Table 1: Determination of HPR: Antibody (or Protein) Molar Ratio

MW of Antibody or Protein	Amount of Antibody or Protein	Molar Ratio of HRP: Antibody (or Protein)
< 5 kDa	0.05 – 1.0 mg	1:1 to 1:10
~150 kDa	0.1 mg or less	10:1
~150 kDa	> 0.1 mg	5:1
> 700 kDa	0.05 – 1.0 mg	25:1

Part 2: Determine the amount and volume of SureFIRE Activated HRP for conjugation reaction

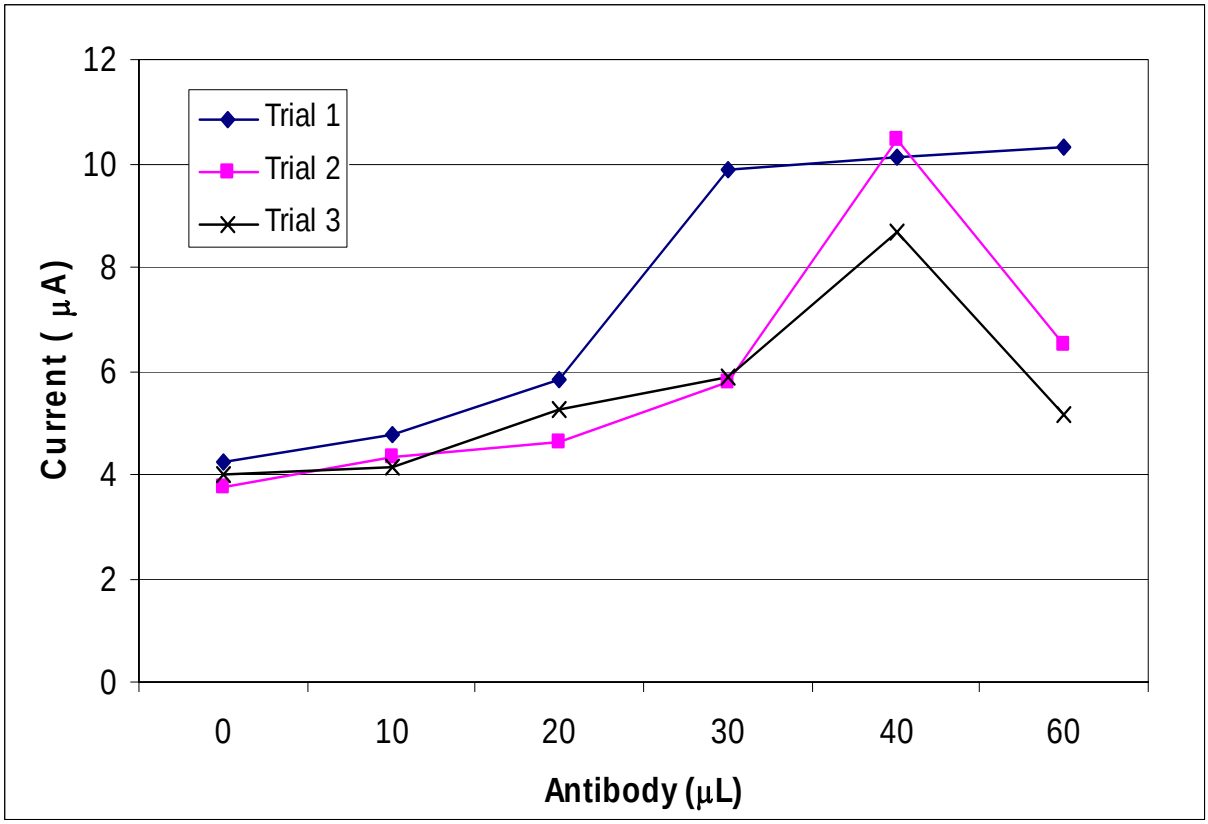
To determine the amount of SureFIRE Activated HRP to use in Step 2 of the Conjugation Protocol, use the molar ratios described in Part 1. Table 2 provides examples of common antibody sample amounts and the volume of Activated HRP required.

Table 2:.Calculating Required Volume of SureFIRE Activated HRP

Molar Ratio of HRP:IgG (Determined by Table 1)	IgG Sample Amount MW 160kDa	Required HRP MW 44kDa	Rehydration of Activated HRP with HRP Conjugation Buffer and Choices of Products	Required Volume of Activated HRP
10:1	0.05 mg (0.3 nmole)	0.15 mg (3 nmole)	Add 200 µL of buffer to 1 vial of 0.3 mg HRP (84-01-01)	100 µL
10:1	0.10 mg (0.6 nmole)	0.3 mg (6 nmole)	Use 1 vial of 0.3 mg HRP (84-01-01) in powder form	Add IgG solution directly to HRP vial
5:1	0.25 mg (1.5 nmole)	0.38 mg (7.5 nmole)	Add 200 µL of buffer to 1 vial of 1.5 mg HRP (84-01-02)	50 µL
5:1	0.50 mg (3.0 nmole)	0.75 mg (15 nmole)	Add 200 µL of buffer to 1 vial of 1.5 mg HRP (84-01-02)	100 µL
5:1	0.75 mg (4.5 nmole)	1.13 mg (22.5 nmole)	Add 200 µL of buffer to 1 vial of 1.5 mg HRP (84-01-02)	150 µL
5:1	1.00 mg (6.0 nmole)	1.5 mg (30 nmole)	Use 1 vial of 1.5 mg HRP (84-01-02) in powder form	Add IgG solution directly to HRP vial

Note: The calculations use molecular weight of IgG = 160 kDa and molecular weight of HRP = 44 kDa. Other antibody amounts and proteins with varying molecular weights can be used with this kit.

APPENDIX C
PHASE 1 ANTIBODY VOLUME VERSUS CURRENT


12
10
8
6
4
2
0
Current (μA)
Trial 1
Trial 2
Trial 3
0
10
20
30
40
60
Antibody (μL)

VITA

Danyelle Small was born in Bunkie, Louisiana, a small town outside Marksville, Louisiana, where her family grew up. She was raised in New Orleans, Louisiana, where she first began an interest in biological engineering. She then obtained her Bachelor of Science in biological engineering degree at Louisiana State University in May 2003. She remained at Louisiana State University for her Master of Science in biological engineering degree. She is currently looking to apply her background to a career in industry or consulting.