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Boron Doped Diamond Biosensor for Detection of *Escherichia coli*

EHSAN MAJID,[†] KEITH B. MALE,[†] AND JOHN H. T. LUONG*[,†,‡]

Biotechnology Research Institute, National Research Council Canada, Montreal, Quebec, Canada H4P 2R2; and Department of Chemistry, University College Cork, Cork, Ireland

o-Nitrophenol, released from *o*-nitrophenyl-β-D-galactopyranose as catalyzed by β-galactosidase, a tetramer of *Escherichia coli*, has been exploited for the detection of *E. coli* contamination in foodstuffs. This reaction was detected using a boron doped diamond electrode poised at +0.93 V, without any surface modification. The enzyme was effectively induced by isopropyl-β-D-thiogalacto-pyranoside with the maximum enzyme activity observed with sodium dodecyl sulfate at 50 °C. A biphasic calibration plot was observed: 4×10^4 to 2×10^5 cells/mL and 2×10^5 to 6×10^6 cells/mL for the first and second region, respectively. The detection limit was 4×10^4 cells/mL with a total analysis time of <1.5 h. Electrode fouling was easily overcome by ~40 rapid CV scans, and the method was applicable for detecting *E. coli* in artificially spiked samples of beef, pork, chicken, milk, and tap water.

KEYWORDS: Electrochemical detection; boron doped diamond; *E. coli*; β-galactosidase; *o*-nitrophenyl-β-D-galactopyranose

INTRODUCTION

About 5000 people die each year from *Salmonella* and/or *Escherichia coli* induced food poisoning in the United States (*1*). The presence of *E. coli* in foodstuffs and drinking water is a chronic worldwide problem as reflected by Canada's worst-ever outbreak of *E. coli* contamination where seven people died and hundreds suffered from the ill effects of drinking contaminated water. This food-borne adulterant can cause hemorrhagic ulcerative colitis and hemolytic uremic syndrome. An *E. coli* outbreak in Scotland infected 260 people and caused 17 fatalities (*2, 3*). The largest recall of food product contaminated with *E. coli* O157:H7 occurred in the summer of 1997, when 25 million pounds of hamburger meat was recalled by a single meat-processing company (*4*). Consequently, the environmental and food industries are potentially emerging markets for biosensing and monitoring of pathogens. The worldwide food production industry is worth about U.S. $578 billion, and the demand for biosensors to detect pathogens and pollutants in foodstuffs is expected to grow in the near future (*5*).

The diagnosis of food contamination would greatly benefit from new methods that combine better sensitivity and speed than existing ones such as polymerase chain reaction (*6*) and immunoassays (*7*), which in many cases are quite time-consuming with multiple steps involved. A fluorogenic detection method for *E. coli* has been developed based on the cleavage of methylumbelliferyl-D-glucuronide (MUG) (*8*). The sensor detected 5×10^7 colony forming units (CFU) per mL in less than 30 min; however, some strains of *Salmonella*, *Shigella*, and *Yersinia* are also capable of splitting MUG; the latter two genera are usually not present in food. A disadvantage is that enterohemorrhagic *E. coli* (EHEC) strains are generally negative in this test. Automated or semiautomated systems are also being used for the detection of *E. coli* as part of the detection methods for *Enterobacteriaceae.* A biosensor using B lymphocytes has been engineered to emit light within seconds of exposure to specific bacteria including *E. coli* (*9*). Techniques involving impedance measurements have shown promise. Yang et al. (*10*) reported an interdigitated array microelectrode based label free electrochemical impedance immunosensor with a detection limit of 10^6 CFU/mL for *E. coli.* Other techniques such as immunoassays and nucleic acid hybridization studies used to enumerate *E. coli* and direct DNA probes at a number of genes have also been developed. An avidin polyaniline modified platinum disk electrode with a 5′-biotin labeled probe immobilized on the surface was used for *E. coli* detection using differential pulse voltammetry in the presence of methylene blue as a DNA hybridization indicator (*11*). Although these techniques possess better detection limits ($<10^3$ CFU/mL), they are associated with antibody/antigen labeling or complimentary DNA binding schemes, which make them less attractive for direct pathogen detection in food and water samples, since such techniques would require the isolation of pathogens from the sample and additional analysis steps to make available appropriate biomolecules or genetic material for the detection step.

E. coli detection using cathodic amperometry on a bismuth nanoparticle modified GC electrode integrated to a flow injection system has been recently reported by Zhang et al. (*12*). A detection limit of 100 CFU/mL and an analysis time of 3 h were reported; however, no food samples were challenged. In

* To whom correspondence should be addressed. E-mail: john.luong@cnrc-nrc.gc.ca. Fax: 514-496-6265.

[†] National Research Council Canada.

[‡] University College Cork.

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E. coli, β-galactosidase is produced by activation of the *lac* operon, as the *lacZ* gene. This hydrolase catalyzes β-galactosides into monosaccharides as the β-linkage of the substrate is cleaved at the terminal carboxyl group on the side chain of a glutamic acid. Monovalent potassium ions (K^+) as well as divalent magnesium ions (Mg^{2+}) are required for the enzyme's optimal activity. Like lactose, ONPG is a molecule composed of two rings held together by an oxygen bridge, which can be hydrolyzed by β-galactosidase. The color intensity of the yellow product released, ONP, serves as an indicator of the hydrolysis rate.

In this study, the basis for the rapid testing for the presence of *E. coli* is the hydrolysis of ONPG by β-galactosidase, since most *E. coli* strains possess this enzyme. ONP was detected electrochemically using a boron doped diamond (BDD) electrode without electrode modification to ascertain the presence of *E. coli* without any pretreatment steps.

MATERIALS AND METHODS

Materials and Chemicals. *Escherichia coli* ATCC 11303 and 10798(K-12) were purchased from American Type Culture Collection (ATCC, Manassas, VA). β-galactosidase, 2-nitrophenol, lysozyme, isopropyl-β-D-thiogalacto-pyranoside (IPTG), *o*-nitrophenyl-β-D-galactopyranose (ONPG), and sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich (St. Louis, MO). Sodium chloride, potassium phosphate (mono- and dibasic), and sodium hydroxide were obtained from EMD chemicals (Gibbstown, NJ). Agar, tryptone, and yeast extract for *E. coli* cell cultivation were obtained from Becton Dickinson (Cockeysville, MD). All solutions were prepared using Milli-Q (Millipore, Bedford, MA) A-10 gradient deionized water (18 MΩ cm). Food samples of beef, pork, chicken, and milk were obtained from the local market.

Cell Growth and Sample Preparation. Lyophilized *E. coli* was reconstituted in sterile LB broth consisting of 10 g of tryptone, 5 g of yeast, and 10 g of NaCl per liter, adjusted to pH 7 with 1 M NaOH. The culture was grown overnight (15−18 h) at 37 °C in a shaker (Innova 4330, New Brunswick Scientific, Edison, NJ) at 250 rpm until an absorbance of 1 at 600 nm was obtained (Beckman DU 640 spectrophotometer, Fullerton, CA). Resulting cells were chilled on ice, centrifuged (Eppendorf 5810R, rotor no. A462) at 3200*g* for 15 min, and washed 3 times before resuspending in 30 mL of 10% sterilized glycerol. This suspension was aliquoted (500 μL) and stored at −80 °C.

Induction of β-galactosidase activity was performed by thawing on ice an aliquot and resuspending 100 μL of the cell suspension in 15 mL of LB broth. The resulting solution was induced by IPTG (150 μL, 50 mM) and incubated at 37 °C for 30 min with shaking at 250 rpm. After centrifugation, the cell pellet was resuspended in 1 mL of buffer and kept on ice for further use. To determine the cell concentration, a series of dilution steps was performed to reduce the cell concentration 10^7-fold. A 100 μL of sample was transferred and spread on an agar plate and allowed to grow overnight, with the number of colonies counted used to determine the cell concentration. Induction was carried out on a daily basis after it was noted that the enzyme activity decreased significantly within 90 days if the cells were frozen. The optimum IPTG concentration for cell induction was evaluated between 0.05 and 5 mM.

For meat sample preparation, 100 μL of cell suspension was mixed with 0.5−1 g of ground meat and transferred to a centrifuge tube. LB broth (15 mL) was added to this mixture, which was then homogenized at 3000 rpm for 30 s, induced, and incubated as previously specified. After incubation, the mixture was centrifuged, and the homogenate residue was resuspended in buffer to a final volume of 1 mL and kept on ice for electrochemical experiments. For milk and tap water experiments, samples were directly spiked with *E. coli* after induction.

Electrochemical Detection of *O*-Nitrophenol. Different electrochemical techniques were performed using a multichannel potentiostat (model 1030, CH Instruments, Austin, TX) for 2-nitrophenol detection, including anodic amperometry, cathodic amperometry, and differential pulse voltammetry using either a BDD (3 mm, 0.1% doped diamond, Windsor Scientific, Slough, Berkshire, U.K.) or a glassy carbon (GC, 3 mm, BAS, West Lafayette, IN) working electrode. A Pt wire (Aldrich, 99.9% purity, 1 mm diameter) and an Ag/AgCl (3 M NaCl) electrode (BAS, West Lafayette, IN) were used as the counter and reference electrodes, respectively. The detection was performed in a 10 mL volume with 50 mM phosphate, pH 7, or 50 mM Britton-Robinson (B-R), pH 4, buffer.

Optimization of β-Glycosidase Reaction. Enzyme activity was monitored before performing any electrochemical measurements. The assay was performed by adding β-galactosidase (0.5 U/mL) to ONPG in 50 mM phosphate pH 7.0 and observing the absorbance at 420 nm as a function of time. ONPG concentrations were varied from 0.1 to 50 mM. Absorbance measurements exhibited a linear relationship from 0.1 mM to 2 mM for *o*-nitrophenol.

For electrochemical measurements, initial attempts were made to detect *o*-nitrophenol in LB media; however, no response was observed. Amperometric *i-t* curves of 4 μM additions of *o*-nitrophenol in 50 mM buffer containing individual components of the LB broth revealed that the presence of tryptone and yeast extract inhibited the detection of *o*-nitrophenol. Therefore, all experiments were performed in phosphate buffer. To monitor the enzyme activity, a certain amount of enzyme was introduced to the electrochemical cell. The rate could be calculated from the current change from the point of injection and onward on the *i-t* curve. The enzyme concentration was optimized over the range 0.01−1.0 U/mL, using 1 mM ONPG (10 mL) in buffer with the rates linear throughout such concentration ranges. Similar experiments were performed to obtain rates at different substrate concentrations (0.1−1.0 mM). The pH optimum was also determined in the range 4−8.

Optimization of Electrochemical Detection of *E. coli*. *E. coli* strains were detected by spiking a known number of cells into an electrochemical cell containing 10 mL of 1 mM ONPG in buffer. The SDS concentration was optimized between 0 and 0.5 mg/mL. The electrochemical cell for temperature experiments consisted of a water-jacketed cell vial (model 3770, BAS, West Lafayette, IN) connected to a thermostatted water bath, and the effect of temperature was monitored between 20 and 50 °C. The vial temperature reached equilibrium within 5 min, and efficient heating/mixing of the sample was accomplished with a hot plate/stirrer. Upon the level-off of the *i-t* curve, the substrate in the vial was spiked to a cell concentration of 3×10^6 cells/mL. All cell dilutions were made in the working buffer. The stability of the BDD electrode was verified by running consecutive cell assays with and without 40 cycles of CV cleaning in between each run. The potential fouling was confirmed from the CVs performed with 1 mM $K_3Fe(CN)_6$.

RESULTS AND DISCUSSION

Electrochemical Characteristics of *O*-Nitrophenol Detection with the BDD Electrode. Cyclic voltammetry of *o*-nitrophenol in 50 mM phosphate buffer pH 7 (**Figure 1**) indicated a well defined oxidation peak at 0.93 V and a second smaller one at 0.4 V. Amperometric detection at +0.93 V (**Figure 1**, **inset**) of 2 μM increments of 2-nitrophenol resulted in good reproducibility and insignificant fouling after nine consecutive additions. Detection sensitivity was 0.187×10^{-7} A/μM with a detection limit of 320 nM ($S/N = 3$). Electrode polishing was not mandatory in between consecutive runs; however, rapid cyclic voltammetry was carried out to minimize fouling effects.

Although differential pulse voltammetry could have been used to detect *o*-nitrophenol, it required several linear voltage sweeps in between runs to remove the entire deposited sample from the electrode. Amperometric detection of nitrophenols could be performed with a GC or GC modified electrode in the B-R buffer system at pH 4 using cathodic conditions. However, in terms of overall stability and sensitivity, anodic amperometry using a BDD electrode in phosphate buffer between pH 6 and 7

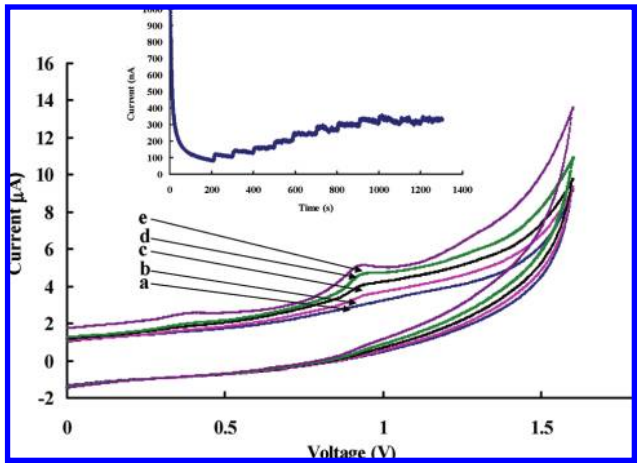

Figure 1. Cyclic voltammograms of *o*-nitrophenol (μM) in 50 mM phosphate buffer, pH 7: (**a**) 0; (**b**) 8; (**c**) 16; (**d**) 32; (**e**) 64. A boron doped diamond (BDD) working electrode (3 mm) was used at a scan rate of 50 mV s^{-1}, with Ag/AgCl and Pt as reference and counter electrodes, respectively. (**Inset**) Indicates repeated injections of 2 μM *o*-nitrophenol at +0.93 V.

produced the best response (figure not shown). Compared to the GC electrode, the BDD electrode signal was enhanced 2-fold for analyte detection at pH 7. As discussed later, β-galactosidase showed higher activity at higher pH.

Effect of ONPG, SDS, and IPTG Induction Concentrations and Temperature on the β-Galactosidase Reaction. Any factors that influence the diffusion process, cell porosity, or amount of reactants will affect the rate. The reaction rate determined spectrophotometrically increased with an increase in ONPG. A similar electrochemical pattern was observed; hence, a concentration of 1 mM was used for further experiments. Before cell experiments could be performed, the cells had to be incubated at 37 °C for 30 min in the presence of IPTG. The rate for the electrochemical assay with 3×10^6 cells/mL increased with increasing IPTG concentration and became constant at 1 mM (**Figure 2A**). Hence, further experiments were performed using 0.5 mM IPTG. At low concentration, SDS degrades the plasma membrane of *E. coli*, causing greater porosity of the cell wall (*13*) or membrane rupture. An 8-fold increase in the rate was observed by increasing the SDS concentration from 0 to 0.01 mg/mL; however, no significant increase was monitored above 0.05 mg/mL (**Figure 2B**). Thus, consequent experiments were performed using an SDS concentration of 0.05 mg/mL. Incubation temperature of *E. coli* has a marked effect on the structure of the cell wall (*14*). Higher temperature incubation leads to a weaker structure of the cell wall, which would be advantageous for diffusion. The β-galactosidase enzyme showed thermophilic behavior when it was assayed with ONPG alone, and a 70-fold rate increase was observed when the cell assay temperature was increased from 20 to 50 °C (**Figure 2C**). Such behavior could be attributed to enhanced enzyme activity at high temperature and/or high porosity of the resulting cell membrane. Higher temperatures (>50 °C) were accompanied by higher noise level and the failure of electrode housing materials; therefore, all experiments were performed at 45 °C. The rate was calculated from the *i-t* curve using the change in current per unit time within the first 3 min after injection. It should be noted that *o*-aminophenyl-β-D-galactopyranose could be an attractive substrate for β-galactosidase since *o*-aminophenol can be detected at lower potentials with high reversible electrochemistry to alleviate electrode fouling. However, it is not clear whether this substrate can migrate through the cell pores and react with β-galactosidase, a subject of future endeavor.

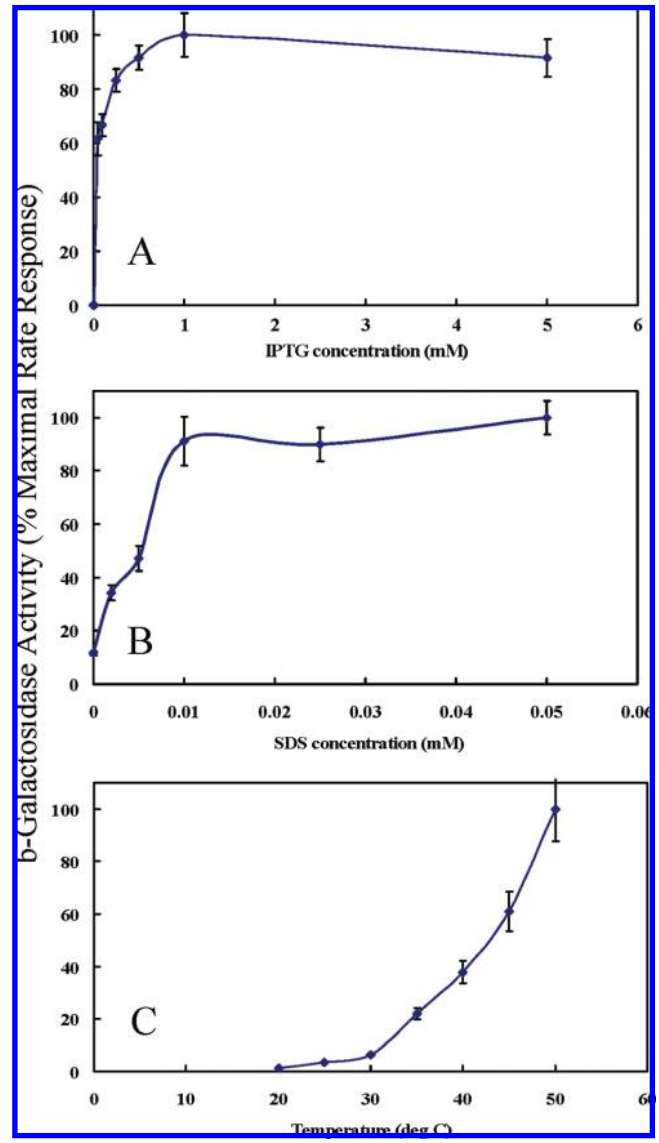

Figure 2. (**A**) Effect of IPTG concentration on the induction of β-galactosidase. *E. coli* cells (3×10^6 cells/mL) were incubated with IPTG for 30 min at 37 °C, and the activity (*o*-nitrophenol production) was then monitored electrochemically in the presence of 1 mM ONPG. Standard error of mean (SEM), $n = 4$. (**B**) Effect of SDS concentration on β-galactosidase activity with induction of *E. coli* (3×10^6 cells/mL) at 0.5 mM IPTG, SEM, $n = 4$. (**C**) Effect of temperature on β-galactosidase activity with induction of *E. coli* (3×10^6 cells/mL) at 0.5 mM IPTG and addition of 0.05 mg/mL SDS, SEM, $n = 4$.

Detection of *E. coli.* Under optimized conditions, **Figure 3** shows a calibration curve for the current rate versus cell concentration for *E. coli* 11303. The curve was biphasic with a linear region between 4×10^4 and 2×10^5 cells/mL with a sensitivity of $(1.38 \pm 0.38) \times 10^{-15}$ (A mL)/(s cell) ($R^2 = 0.9919$; $n = 4$, 95% confidence interval) (**Figure 3**, **inset**). The second region of the curve was linear between 2×10^5 cells/mL and 6×10^6 cells/mL with a detection sensitivity of $(0.88 \pm 0.12) \times 10^{-15}$ (A mL)/(s cell) ($R^2 = 0.9912$; $n = 6$, 95% confidence interval). The detection limit was about 4×10^4 cells/mL. The biphasic nature of the calibration curve was confirmed from repetitive experiments. It should be noted that *E. coli*

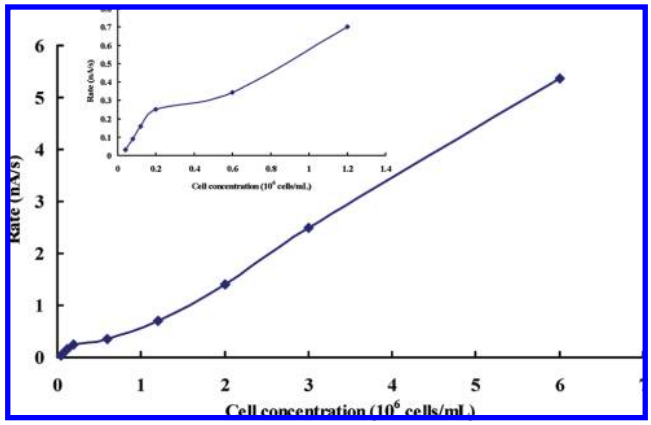

Figure 3. Calibration curve for *E. coli* detection (*o*-nitrophenol production rate) using a BBD working electrode. The β-galactosidase from *E. coli* was induced for 30 min at 37 °C with 0.5 mM IPTG. Electrochemical detection (+0.93 V) was performed at 45 °C in 50 mM phosphate buffer, pH 7, containing 1 mM ONPG and 0.05 mg/mL SDS. The inset shows the lower range of detection between 4×10^4 cells/mL and 1.2×10^6 cells/mL.

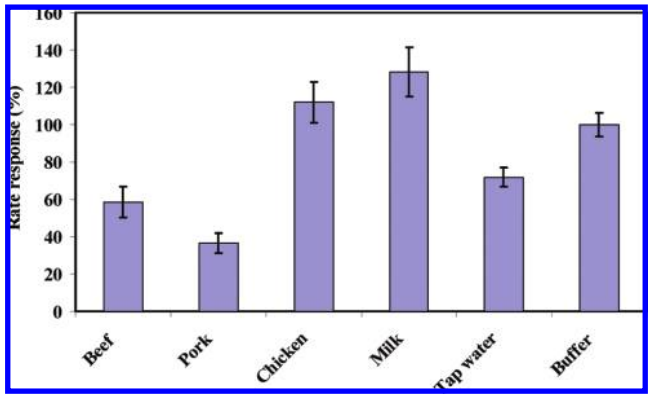

Figure 4. Comparison of various food samples artificially contaminated with *E. coli* (1.2×10^6 cells/mL). The rates obtained were compared to the control (1.2×10^6 cells/mL in buffer) set as 100%. Detection was performed as described in **Figure 3**. SEM, $n = 4$.

10798(K-12) produced a current rate 80% that of strain 11303. Therefore, strain 11303 was used for all subsequent experiments.

Three different meat samples, milk, and tap water were tested for contamination by spiking the samples with *E. coli*. Meat homogenate in buffer was directly spiked into the electrochemical cell to attain a final concentration of cells of 1.2×10^6 cells/mL. Milk and tap water were contaminated to the same extent. **Figure 4** shows the rates for beef, pork, chicken, milk, and tap water samples. The current rates for these curves were determined after an initial large increase in current upon the addition of a homogenate sample. This instantaneous change in current was attributed to an electrochemically active component within the homogenate matrix and was verified by adding different volumes of homogenate without cells into the electrochemical cell during the *i-t* curve. The obtained current increments were proportional to the homogenate volume added for all meat samples. Milk and tap water samples were contaminated with *E. coli* directly from a freshly induced stock. The rate for the chicken (113%) and milk (130%) samples were higher when compared to the buffer. However, in the case of beef, pork, and tap water, the rates were less than for the buffer at 60, 37, and 73%, respectively.

Electrode fouling for the homogenate samples containing cells (**Figure 5c**) was much greater than that of the cell sample

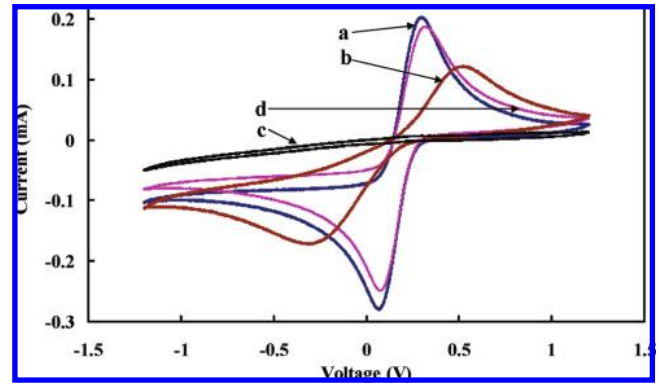

Figure 5. Effect of rapid CV scanning (40 scans) between runs to counter BDD electrode fouling: (**a**) pristine BDD electrode; (**b**) after cell assay without CV scanning; (**c**) after cell assay in the presence of beef homogenate without CV scanning; (**d**) cleaning of electrode surface by CV scanning after cell assay. The CV of the BDD electrode was performed in the presence of 1 mM $K_3Fe(CN)_6$.

(**Figure 5b**), which was partly expected due to electrode fouling by debris and fat. However, upon completion of 40 rapid CV scans (**Figure 5d**), the electrode surface was regenerated, providing a CV profile similar to the pristine BDD electrode (**Figure 5a**). For cell assays the electrode stability was very reproducible for repeated analysis (±7%, 95% confidence interval, $n = 12$). However, if the CV step was not performed between runs, the signal lost 92% of its initial value within the first 3 runs.

In brief, an electrochemical biosensor using a BDD electrode was developed and tested for detecting β-galactosidase of *E. coli* in various food samples and tap water. With a detection limit of 4×10^4 cells/mL and a total analysis time of less than 1.5 h, this procedure compared well with current techniques. Although the sensor was used for beef, pork, chicken, milk, and tap water samples only, it has the potential to be employed in any kind of food or drink sample. Variation in the detection from one kind of sample to another can be avoided by calibrating in the same matrix as the samples. In addition, β-galactosidase is an essential enzyme in the human body, and its deficiency can result in galactosialidosis or Morquio B syndrome. Thus, the β-galactosidase assay is used frequently in genetics, molecular biology, and other life sciences. Boron doped diamond interdigitated microelectrodes could be considered a suitable alternative for highly sensitive detection of multiple pathogens simultaneously by employing more than one enzyme/substrate pair in the detection scheme. This technology can be adapted for field tests to rapidly detect traces of *E. coli* and other foodborne pathogens.

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