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A rapid and selective method for monitoring the growth of coliforms in milk using the combination of amperometric sensor and reducing of methylene blue

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ABSTRACT

A novel method for monitoring the growth of coliforms in milk was developed based on measuring the current change in an amperometric sensor. The sensor consists of a circuit with a homemade potentiostat and a pair of electrodes. The electrode was immersed in milk samples containing methylene blue with various concentrations of bacterial inoculums. The microbial metabolism led to the reduction of methylene blue resulting in a change of current. The time required to identify readily detectable change (detection time, DT) provided an approximate measurement of the amount of microorganisms in the initial inoculums. The sensor system used in this study has the selectivity towards coliform bacteria such as *Escherichia coli* and *Enterobacter aerogenes*. The calibration curve of DT against concentration of coliform showed a linear correlation coefficient ($R^2 = 0.9192$) over the range of 10^2–10^8 CFU/mL. The sensor was able to detect the coliform bacteria at initial concentrations of 10^5 CFU/mL within 6 h, making it suitable for use in real-time monitoring of bacterial growth. This system has potential application in the detection of coliform concentration in milk at dairy farms when a proper selective media is designed.

1. Introduction

Indicator microorganisms may be employed to reflect the microbiological quality of foods relative to product shelf or their safety. The use of coliform counts as an indicator of sanitation has been a common tool in public health protection for many years. Coliforms are a group of bacteria commonly found in the environment, including soil, surface water, vegetation and the intestinal tracts of warm-blooded animals. Typical coliforms include genus *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella* and *Serratia*. Some coliforms such as *Escherichia coli* are generally regarded as the indicators in the case of water and dairy products. Detection of coliforms is used as a general indicator of sanitary conditions in dairy production and processing environments, and thus, their propagation in the food industry is an important issue. Therefore, elevated coliform counts in milk and dairy products suggest unsanitary conditions exist during production, processing or packaging.

The methods for pathogenic bacteria detection are critical to food safety and human health. Culturing and plating method is the oldest detection technique and remains the standard method for detecting pathogenic bacteria. However, other techniques are necessary because culturing methods are excessively time-consuming. It may take up to 7 or 8 d to yield an answer and thus is obviously inconvenient in many industrial applications, particularly in foods [1]. Various methods have been developed for the rapid determination of bacterial counts in the recent decades, including ATP estimations [2], direct epifluorescent filter technique [3], methylene blue reduction method [4] and electrical methods [5,6]. Among the methods, the ATP method and epifluorescent filter method are fast but complicated. Methylene blue has been used for bacterial enumeration. The solution is blue until it is reduced and turns colorless [7]. However, methylene blue reduction method lacks of selectivity for microbial detection.

Impedance method [8] was one of the earliest electrical methods for the detection of bacteria in foods, and has been developed as a rapid method that could detect bacteria within 24 h [9–14]. Despite their widespread application, these techniques have many disadvantages including polarization of the probe electrodes, decreased sensitivity of the device in more conductive media and high instrument costs [15–17]. Although some new technologies meet sensitivity requirements their analysis times are lengthy. For example, polymerase chain reaction (PCR) can be used to amplify a small amount of genetic material to determine the presence of

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bacteria but requires hours of processing [18,19]. In addition, the method required complicated preparing procedure and needed expensive instrument. In our previous study, the signal of electrode impedance change was converted to frequency change using a series piezoelectric quartz crystal (SPQC) sensor. This crystal system is characterized by its low cost and compactness. However, with higher initial bacterial concentrations in solution, a greater frequency drop and more rapid frequency fluctuation were observed such that all curves could not be expected to originate at the same frequency [20]. Amperometric sensors are very attractive alternatives because they can provide specific and repetitive assays using miniaturizable, robust, and inexpensive devices [21,22]. Composed of an enzyme-modified screen-printed carbon strip, amperometric sensors have been developed and commercialized [23]. The electroactive species generated during the enzymatic reaction are measured by amperometric sensors. The most important advantage of amperometric sensors is that they can be applied in colorful and turbid media [24]. An amperometric method in microbial detection is found in the work of Abdel-Hamid et al. [25], where *E. coli* was detected within 30 min at concentrations between 100 and 600 cells/mL using a flow-through immunofiltration method coupled to amperometry [1]. Despite the progress achieved in recent years, there are still few practical sensors for microbial detection which can adequately satisfy market requirements, such as short analysis time, cost-effective instrumentation, high sensitivity and feasibility for on-line monitoring of industrial processes and environmental surveying [26].

Traditionally, the formation of reducing substances during bacterial metabolism causes the color to disappear (e.g. methylene blue reduction test). Thus, the time of reduction is taken as a measure of the amount of organisms in milk although it is more truly a measure of the total metabolic reactions proceeding at the cell surface of the bacteria. The methylene blue reduction test has lost much of its popularity because it requires the color to be recorded at half an hour intervals. The variation is relatively high due to individual visual identification. Therefore, methylene blue reduction was not specific enough because the bacterial distribution is different among dairy farms. A more selective method was required for the measurement of bacterial count in milk. In this study, we combined amperometric sensor with methylene blue reducing to investigate the reducing reaction caused by individual bacterial metabolic reactions. The feasibility of the sensor was studied and it was found that the fabricated sensor was suitable for monitoring the growth of coliforms in milk.

2. Materials and methods

2.1. Reagents and materials

Commercial aseptic milk (Tetra Pak, 300 mL) samples purchased from a local market (Hsinchu, Taiwan) were used to perform the amperometric test. Cultures of *Bacillus subtilis*, *Citrobacter* sp., *E. coli*, *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Lactobacillus* sp., *Staphylococcus aureus*, *Salmonella typhimurium*, and *Saccharomyces* sp. were obtained from the Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan) were inoculated into nutrient broth (Difco) and incubated at 37 °C overnight. Aliquots of the bacteria dilutions were spread on pour plates and incubated at 37 °C for 24 h for enumeration. All colonies were counted after 24 h they were incubated.

2.2. Apparatus

A schematic of the experimental set-up is shown in Fig. 1. Amperometric measurements were performed using a laboratory-built potentiostat. Input and output signals from the potentiostat were coupled to a PC (Pentium 1.8 GHz) using a peripheral interface card (AT-MIO-16E, National Instruments, Austin, TX, USA). The interface card consisted of a 16-channel analog-to-digital (A/D) converter (12 bit) and a 2-channel digital-to-analog (D/A) converter (12 bit). Data display and recording were programmed using the LabVIEW 7.1 software package (National Instruments, Austin, TX, USA). All measurements were taken with a two-electrode system using a pair of planar platinum strips as electrodes (Malthus, C6243; Crawleg, UK). Amperometric measurements were performed at 0 mV.

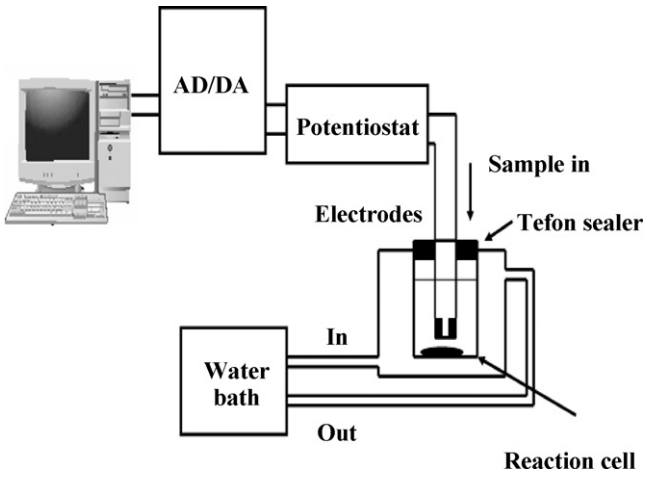

Fig. 1. Schematic diagram of the experimental set-up for monitoring the growth of bacteria.

2.3. Methylene blue reduction procedure

The methylene blue solution was prepared by mixing the methylene blue dye with distilled water containing 5 mg of methylene blue dye per 100 mL. The methylene blue solution was prepared with boiled distilled water on the day of use and protected from exposure to light. The methylene blue solution was filtered through a 0.45 μm filter to make it aseptic. The aseptic milk sample was pre-warmed at 37 °C for 10 min prior to the assay. 9 mL of aseptic milk was mixed in a sterile Malthus glass tube containing 1 mL of a methylene blue solution. Then, 0.1 mL samples of *E. coli* at various dilutions were added to sterile tubes. The prepared samples were then immediately incubated at 37 °C and examined at 30 min intervals. Methylene blue is blue in color at oxidized status and becomes colorless at reduced status due to the metabolic activity of bacteria.

2.4. Amperometric measurement procedures

The reaction cell was constructed from a glass tube with a Teflon cover lid which held two identical electrodes. To eliminate cross-contamination, the reaction cell and electrodes were washed and rinsed with distilled water and sterilized at 121 °C for 15 min before each incubation and prior to immersion in any of the solution samples. The commercial sterilized milk was added into the Malthus glass tubes up to the level of the electrodes. The total sample volume was 10 mL for each measurement. A 0.1 mL sample of *E. coli* (incubated overnight at 37 °C then diluted to various concentrations) and 1 mL methylene blue solution were spiked into the sterilized milk. Amperometric measurements were performed using a laboratory-built potentiostat. Input and output signals from the potentiostat were coupled to a PC using a peripheral interface card (AT-MIO-16E, National Instruments, Austin, TX, USA). The interface card consisted of a 16-channel analog-to-digital (A/D) converter (12 bit) and a 2-

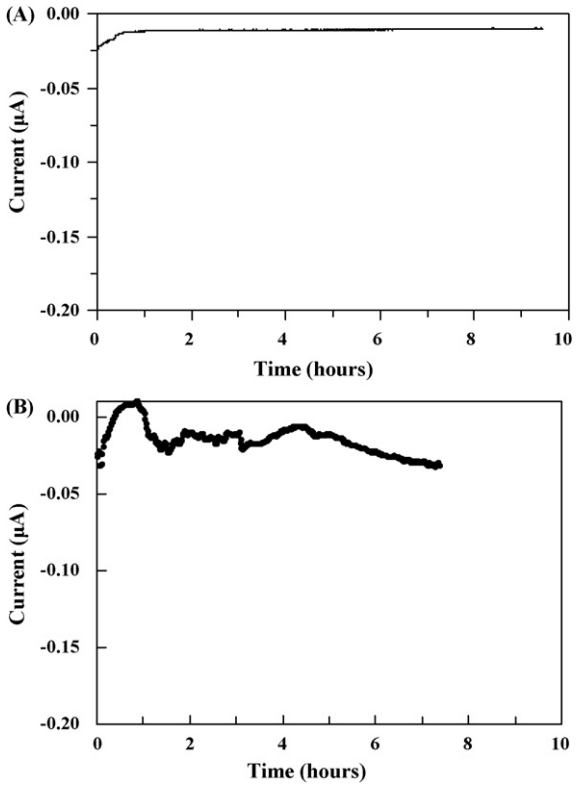

Fig. 2. Current profile of the amperometric sensor when the electrode was immersed into the milk sample. Measurements were performed at 37 ± 0.2 °C with a sample volume of 5 mL. (A) Aseptic milk sample containing 5% methylene blue and (B) milk sample inoculum 10^8 CFU/mL *E. coli* without methylene blue.

channel digital-to-analog (D/A) converter (12 bit). Voltage output, data display and recording were programmed using the LabVIEW 7.1 software package (National Instruments, Austin, TX, USA). The sensor was employed continuously for 10 h to measure the current change in the solution sample. The reaction cell was fully covered with aluminum foil to prevent a reduction reaction caused by the light during measurement. The amperometric measurement was performed at 0 mV. The current change was displayed and recorded by the computer until the incubation period was completed.

3. Results and discussion

3.1. Current profile of the detecting sensor in milk samples

The time from the beginning of the measurement until a rapid increase in current at cathodic direction was observed and the DT was inversely related to the initial concentration of bacteria. The time required for obtaining a current drop more than 0.05 μA was reported as the detection time (DT). The bacteria count determined by the plate count method against the DT was plotted as a calibration curve. Two experiments were conducted to determine the influence of methylene blue and *E. coli*, respectively, on the fluctuation of current detected by the sensor. First, 1 mL of methylene blue solution was added into a 9 mL sterilized milk sample and the electrodes were immersed into the sample to detect the current profile. As shown in Fig. 2(A), the current increased dramatically in the initial period (ca. 60 min), then reached a steady state throughout the period of bacterial growth until a threshold time was reached. Thereafter, the response of the sensor remained stable for more than 9 h. The noise level of the sensor was ±0.005 V over an average period of 9 h. This result indicated that the current of the sensor was very stable if there was no chemical or physical influence induced by

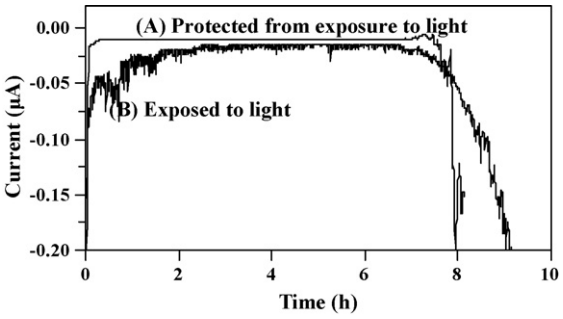

Fig. 3. Effect of light on the current response curves at 37 °C using the sensor. The electrode was immersed in commercial aseptic milk with *E. coli* inoculums 10^3 CFU/mL. (A) Reaction cell protected from exposure to light and (B) reaction cell exposed to light.

microbial growth in milk. Second, the current profile was observed by detecting the current change in a milk sample which was inoculated 10^8 CFU/mL of *E. coli* but without methylene blue solution. As shown in Fig. 2(B), the noise level of the sensor was ±0.005 V over a period of 8 h. This result indicated that the microbial growth of the milk aroused the current of the sensor only to a negligible level. Based on these results, it can be concluded that no current was aroused when only either microorganism or methylene blue was present in the sample system.

3.2. Influence of light exposure and volume of methylene blue solution on current response of the sensor

An experiment was carried out to investigate the influence of light exposure on the response of the sensor. As shown in Fig. 3(A) and (B), light exposure affected the DT only at a negligible level, despite the fact that methylene blue could be activated by light to an excited state. The influence of volume of methylene blue on current response of the sensor was also examined. Volumes of 1, 5 and 10 mL had almost identical current profiles (data not shown). These results indicated that the volume of methylene blue solution caused a current change only at a negligible level within the range in this study. An operation volume of 1 mL was selected for the following tests.

3.3. Microbial specificity using the amperometric sensor and methylene blue reducing method

As illustrated in Fig. 4, in addition to current profiles for coliforms (*Citrobacter* sp., *E. coli*, *Ent. aerogenes*, and *K. oxytoca*), those of many bacteria including *B. subtilis*, *Lactobacillus* sp., *Saccharomyces* sp., *Salm. typhimurium* and *Staph. aureus* were also determined. These microorganisms comprise species of genera that are normally associated with milk [27]. Except for *E. coli* and *Ent. aerogenes*, other bacteria could only produce a negligible current change within the detection time. An obvious change of current at the 6th hour was observed for *E. coli* and *Ent. aerogenes*; whereas only an insignificant change of current was found for *Citrobacter* sp. and *K. oxytoca*. Unable to monitor *Citrobacter* sp. and *K. oxytoca* in milk is not a problem because they comprise only a small percentage among the contaminant microbes. The results demonstrated that the growth of coliforms could be monitored using the sensor. In the other way, *Lactobacillus* sp. grew more slowly than coliforms in milk and showed an obvious change of current after 8 h of incubation. Thus, detection of coliforms in milk will not be interfered by *Lactobacillus* sp. Since *E. coli* is more indicative of fecal pollution than the other genera and species noted in the coliform population (especially *Ent. aerogenes*), it is used as the major test strain in the following study.

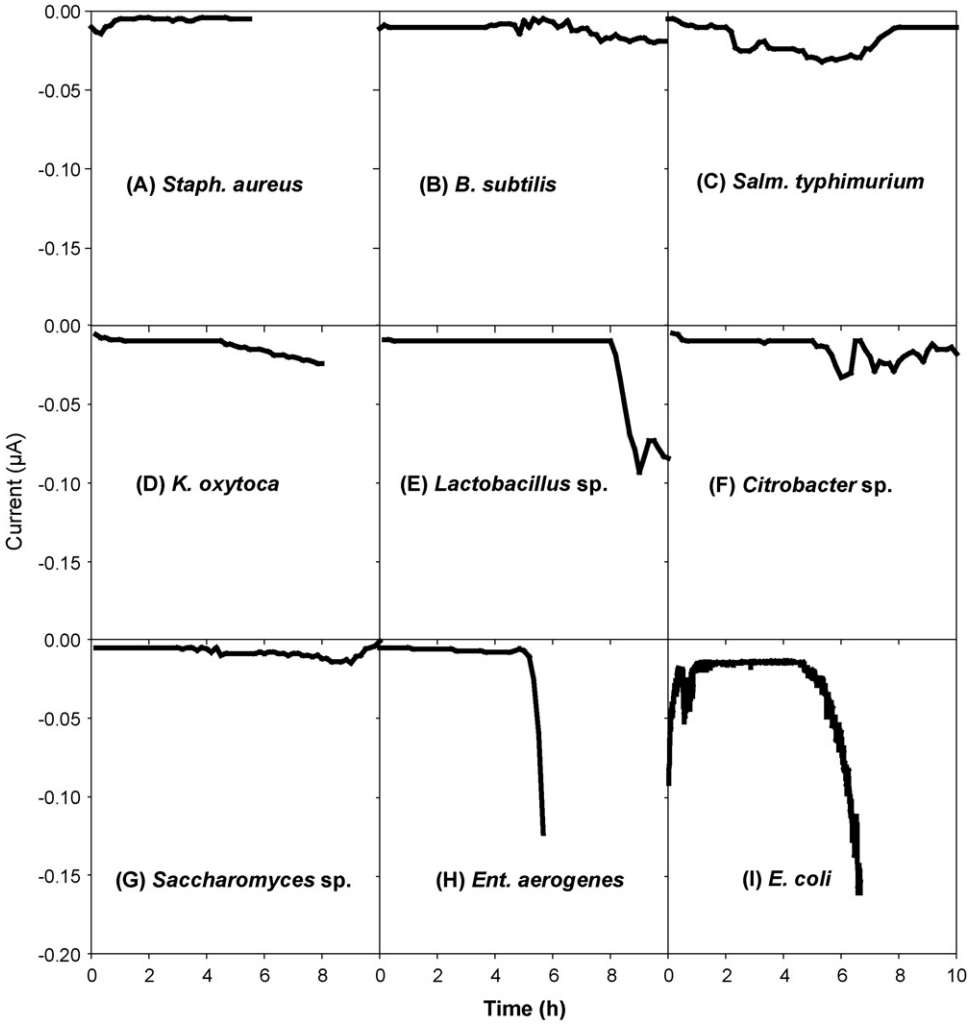

Fig. 4. Current profile of the amperometric sensor when the electrode was immersed in commercial aseptic milk with (A) *Staph. aureus*, (B) *B. subtilis*, (C) *Salm. typhimurium*,(D) *K. oxytoca*, (E) *Lactobacillus* sp., (F) *Citrobacter* sp., (G) *Saccharomyces* sp.,(H) *Ent. aerogenes*, (I) *E. coli*, inoculums of 10^5 CFU/mL. Measurements were performed at $37 \pm 0.2\,^{\circ}C$ with a sample volume of 5 mL. Aseptic milk sample contains 5% methylene blue.

3.4. Detection time (DT) versus standard plate count

Fig. 5(A), (B) and (C) shows example plots of the current response and growth curve of an *E. coli* culture at concentrations of 10^3, 10^5 and 10^8 CFU/mL, respectively. The growth and the current response against time after *E. coli* inoculation were studied. The time required to identify readily detectable changes, which is DT as previously described, provided an approximate measure of the amount of microbial organisms in the initial inoculums. The change in current could be explained by the Nernst equation, which gives the electrode potential (E) relative to the standard electrode potential (E^0) of the electrode couple or, equivalently, of the half cells of a battery. At room temperature the following is true.

$$E = E^0 - \frac{0.0591}{n} \log \frac{[\text{red}]}{[\text{ox}]}$$

where n is the amount of electrons transferred in the half-reaction, [red] is the concentration of oxidizing agent (the reduced species) and [ox] is the concentration of reducing agent (the oxidized species). In this experiment, methylene blue$_{[ox]}$ was reduced to methylene blue$_{[red]}$ due to the microbial metabolism when a 0 mV potential was applied to the electrode. The decrease of electrode potential was caused by the increase of reduced compound, as a result of the decrease in current. The Nernst equation could be applied to explain the current decrease with the extension of time after a threshold time. The elapsed time before the current began to decrease was related to the initial amount of microbial organisms in the milk sample. Fig. 6 shows scattergrams of the logarithm of the initial bacterial concentration of the samples against DT. The data revealed a linear relationship when the log of the bacterial concentration (from 10^2 to 10^8 CFU/mL) was plotted against the frequency DT. The regression equation was $Y = -0.7669X + 8.2256$ over the range of 10^2–10^8 CFU/mL and the correlation coefficient (R^2) was 0.9192. The graph illustrates the relationship between microbial concentration and DT. The time from the inoculation to the initial small change in current was exponentially related to the concentration of microbial organisms after inoculation. The detection time obtained from the proposed method was 0.5–2.8 h shorter than those obtained by the methylene blue reduction method (data not shown). The results revealed that the proposed method could both rapidly and accurately determine the *E. coli* concentration in milk.

3.5. Comparison of E. coli detection with other methods

Bacterial counts play a major role in determining the sanitary quality of milk. High bacterial counts are responsible for quality defects in pasteurized milk, ultra high temperature sterilization

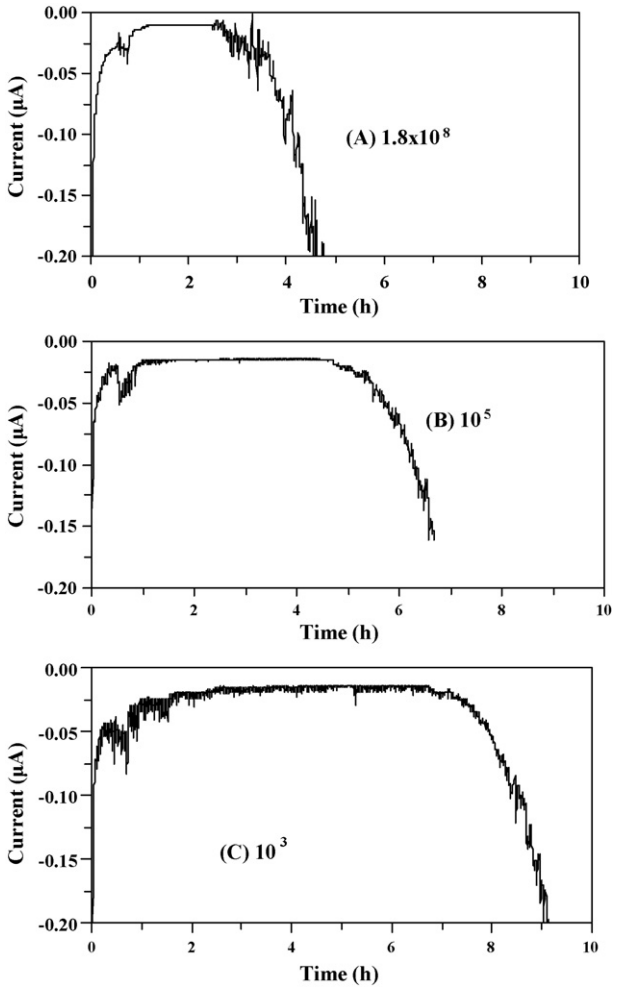

Fig. 5. Current profile of the amperometric sensor when the electrode was immersed in commercial aseptic milk with *E. coli* inoculums of (A) 1.8×10^8 CFU/mL, (B) 10^5 CFU/mL, and (C) 10^3 CFU/mL. Measurements were performed at 37 ± 0.2 °C with a sample volume of 5 mL. The time required for obtaining a current drop more than 0.05 μA was reported as the detection time (DT).

(UHT) milk, whole powder and other milk products [20,28–30]. For this reason, a 10^5 CFU/mL bacterial count upon arrival at the dairy processing plant is the critical limit enforced through Directive 92/46 EEC [31]. The detection method developed in this study

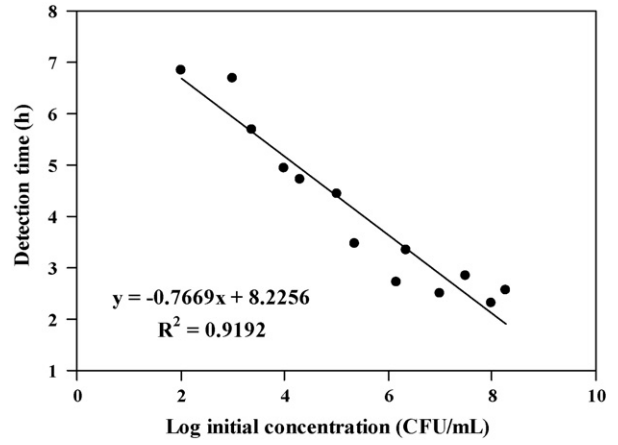

Fig. 6. Scattergram of detection time against the logarithm of the initial bacteria concentration in milk. The sample volume was 5 mL and the sensor was placed in an incubator with the temperature precisely controlled at ±0.5 °C.

was compared with other reported methods. ATP bioluminescence was reported suitable for rapid monitoring of bacterial concentration in raw milk at its reception in the dairy plant [2]. However, the technique and procedures of ATP are much more complex than the method developed in this work. Compared with PCR, this technique takes from 5 to 24 h to produce a detection result but not including any previous enrichment steps. In addition, this method cannot discriminate between viable and non-viable cells [1]. Obviously, the method developed in this study is much easier and the procedures are much simpler than PCR. The ELISA method is based on immunological technique. Daly and Doyle [32] applied PCR–ELISA to determine the concentration of *E. coli* in milk. The DT needed was similar to the method developed in this study, however, our method provides a much broader detection range (10^2–10^4 CFU/mL). The lower detection limit of the technique developed in this study was approximately 100 CFU/mL and the working range was from 10^2 to 10^8 CFU/mL. Thus, the lower detection limit of the amperometric system combined with the redox reaction of the methylene blue to *E. coli* is 1000-fold greater than that of the standard ELISA assay. Additionally, according to the standards set by the U.S. Food and Drug Administration (FDA), the maximum amount of coliform bacteria was at no more than 10 bacteria per milliliter in milk sold raw to the consumer, the same limit required for pasteurized milk. The detection limit using the biosensor system in this study was close to the standards, which make it more practical for rapid detecting coliform bacteria in dairy products. Furthermore, Qu et al. [13] applied SPQC-TAL (Tachypleus amebocyte lysate) technique for detection of *E. coli*. Zhang et al. [17] and Chang et al. [20] also reported the detection of *E. coli* using SPQC technique. Although the DT reported in the studies of Qu et al. [13] and Zhang et al. [17] were faster than for the method developed in this study, the detection limit of our amperometric system combined with the redox reaction of the methylene blue to *E. coli* was 1000-fold greater than their methods. The DT reported by Chang et al. [20] was much longer than for our sensor. Furthermore, interference with the SPQC technique occurs easily and causes a higher noise level such that it exhibits relatively low stability compared with the amperometric system. The electrochemical techniques are much easier to use and have much lower equipment costs than optical ones. Wide concentration range for bacteria detection, relatively low cost of equipment and friendly experiment procedure for user make this method as an applicable candidate for a preliminary screening of coliform in dairy products.

4. Conclusion

This study developed a novel design for monitoring *E. coli* in milk using an amperometric sensor and methylene blue reducing method. The system was able to determine the viable *E. coli* concentration by detecting the current change caused by the oxidation–reduction reaction of methylene blue due to the metabolism of *E. coli* in the milk sample. The DT could be used to determine the initial bacteria concentration. The major reason for the current decrease after inoculation was considered to be the reducing of methylene blue on the surface of the electrode. Compared with conventional bacteria plating methods, i.e. the impedance method and the ATP estimation method, this technique is comparatively simple and does not require the expertise of highly skilled technicians. Compared with conventional methylene blue reducing methods, this amperometric method is also more accurate and faster. This system is a suitable for use in real-time bacteria monitoring.

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