Fluorescence Spectroscopy for Rapid Detection and Classification of Bacterial Pathogens

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This study deals with the rapid detection and differentiation of Escherichia coli, Salmonella, and Campylobacter, which are the most commonly identified commensal and pathogenic bacteria in foods, using fluorescence spectroscopy and multivariate analysis. Each bacterial sample cultured under controlled conditions was diluted in physiologic saline for analysis. Fluorescence spectra were collected over a range of 200–700 nm with 0.5 nm intervals on the PerkinElmer Fluorescence Spectrometer. The synchronous scan technique was employed to find the optimum excitation ($\lambda_{ex}$) and emission ($\lambda_{em}$) wavelengths for individual bacteria with the wavelength interval ($\Delta\lambda$) being varied from 10 to 200 nm. The synchronous spectra and two-dimensional plots showed two maximum $\lambda_{ex}$ values at 225 nm and 280 nm and one maximum $\lambda_{em}$ at 335–345 nm ($\lambda_{em} = \lambda_{ex} + \Delta\lambda$), which correspond to the $\lambda_{ex}$ of 225 nm, $\Delta\lambda = 110–120$ nm, and $\lambda_{ex} = 280$ nm, $\Delta\lambda = 60–65$ nm. For all three bacterial genera, the same synchronous scan results were obtained. The application of principal component analysis (PCA) to the fluorescence spectra resulted in successful classification of the bacteria by their genus as well as their concentration. The detection limit was approximately $10^3–10^4$ cells/mL for each bacterial sample. These results demonstrated that fluorescence spectroscopy, when coupled with PCA processing, has the potential to detect and to classify bacterial pathogens in liquids. The methodology is rapid (<10 min), inexpensive, and requires minimal sample preparation compared to standard analytical methods for bacterial detection.

Index Headings: Fluorescence spectroscopy; Fluorescence; Synchronous scan; Bacterial pathogens; Principal component analysis; PCA; E. coli; Salmonella; Campylobacter.

INTRODUCTION

The global concern of food-borne illness, particularly from ingestion of food-borne bacteria, necessitates rapid detection within food and environmental matrices. Typically, bacterial cultures, DNA-based methods, and antibody-based detection schemes are used to isolate or identify bacteria. Cultural methodology is time-consuming; bacterial pathogens often occur in low numbers and an enrichment procedure, ranging from hours to days, may be required prior to the application of a rapid detection assay. Polymerase chain reaction (PCR) has been reported as one of the most promising methods for detection of pathogens, particularly Salmonella. However, PCR-based assays must include a means to circumvent the frequent presence of inhibitors, may detect the presence of DNA, which may not be indicative of viable bacteria, and lacks sensitivity unless bacterial numbers are sufficiently high.

Advances in rapid methods such as nucleic acid probe hybridization or immunological assays have reduced the detection time of bacterial pathogens in foods but typically require target microorganism concentrations of approximately $10^3–10^6$ cells/mL or more. Therefore, there is a need for the development of more direct, rapid, and sensitive techniques for detecting bacterial pathogens, particularly in foods.

Recently, there has been an interest in vibrational spectroscopy (Fourier transform infrared (FT-IR), Fourier transform near-infrared (FT-NIR), and Raman) for detection and identification of bacteria. Fluorescence spectroscopy has also been used as an alternative method to identify and differentiate bacteria in clinical and food applications. This technique is based on the intrinsic fluorescence of bacteria. Fluorescence from a biological cell mainly originates from its chemical components. Aromatic amino acid residues (tryptophan, tyrosine, phenylalanine), nucleic acids, and co-enzymes with ultraviolet (UV) excitation are the intrinsic fluorophores.

Some of the previous studies have described the identification and classification of bacteria based on intrinsic fluorescence spectroscopy using various excitation–emission wavelengths. Giana et al. reported the identification of three clinically important species, Escherichia coli, Enterococcus faecalis, and Staphylococcus aureus, using excitation at 410 nm and 430 nm. Leblance et al. reported a good classification of 25 strains of bacteria using excitation at 250 nm. Ammor et al. described the identification of lactic acid bacteria isolated from meat and meat products. Here, three excitation wavelengths (250 nm, 316 nm, 380 nm) were tested, with the best classification obtained with 250 nm excitation. Bhatta et al. reported the characterization of lactic acid bacteria (Lactobacillus sp.) and yeast (Saccharomyces sp.) through their autofluorescence spectra. Shelly et al. reported the identification of Pseudomonas species by excitation–emission matrix (EEM) of their fluorescent pigments that has diffused into the growth medium, which method required an incubation procedure of bacteria in specific medium to produce a suitable characteristic profile.

In this study we have investigated fluorescence spectroscopy combined with multivariate analysis to identify and classify bacterial pathogens. Three different genera of bacteria, Escherichia coli (E. coli), Salmonella, and Campylobacter, were tested in this study, which are the most commonly identified commensal (E. coli) and pathogenic (Salmonella and Campylobacter) bacteria in foods. The optimum excitation–emission wavelengths were determined through a synchronous scan technique and the emission spectra obtained were used for principal component analysis (PCA) to classify the bacteria according to the genus and their concentrations.
MATERIAL AND METHODS

Preparation of Bacterial Samples. Escherichia coli (E. coli), Salmonella, and Campylobacter were provided from the culture collection maintained by the Bacteriological Epidemiology and Antimicrobial Resistance research unit, USDA-ARS, in Athens, Georgia. Bacteria were cultured on blood agar plates and incubated overnight for 24 hours at 37 °C for E. coli and Salmonella and for 48 hours at 42 °C for Campylobacter. To examine the reproducibility, each bacterial culture was grown on subsequent days (for three days) and four samplings were conducted from each culture plate, giving a total of 12 samples for each bacterium. Cell suspension of each bacterium was prepared in 0.85% saline solution and their concentrations determined using a calibration curve based on absorbance at 540 nm. Each bacterial sample was diluted to between 10^7 and 10^8 cells/mL in physiologic saline prior to fluorescence scanning.

Fluorescence Spectroscopy. Fluorescence spectra were acquired using a Perkin-Elmer LS55 fluorescence spectrometer (PerkinElmer, USA) operating with the PerkinElmer FL Winlab (version 4.0) software. The spectrometer is equipped with a xenon discharge light source and a photomultiplier detector and includes a single position cell holder for measuring liquid samples. Each bacterial suspension (3 mL) was placed in a high-grade standard (1 cm square) quartz cuvette (10 mm path length) with a screw cap and put in the cell holder. All measurements of the samples were made at room temperature (20–22 °C).

To determine optimum excitation and emission wavelengths for each bacterium, a synchronous scan technique was used, in which both excitation and emission monochromators are scanned simultaneously with constant wavelength intervals between them. This technique has been used for multi-component analysis of complex samples. For synchronous spectra, the excitation was recorded between 200 and 500 nm and the wavelength interval (Δλ) used was from 10 to 200 nm, in 10 nm increments (Δλ = 10, 20, 30, . . . 200), thus a total of 20 spectra were collected per sample. Fluorescence emission spectra (200–700 nm, 0.5 nm intervals) were recorded with excitation wavelengths at 225 nm and 280 nm, respectively. The scan speed was 240 nm per min; thus, the total time for each spectrum was about 2 min. The excitation and emission slit widths were 10 nm each. Each cell suspension was scanned twice.

Data Processing and Multivariate Analyses. Fluorescence spectra (sp format), as acquired with the spectrometer, were imported into Unscrambler (ver. 9.7, CAMO, Oslo, Norway) through the JCAMP format and then exported to MATLAB (ver. 7.3, Mathworks, Inc., Natick, MA) software. PCA was carried out using the PLS_Toolbox (ver. 4.0, Eigenvector Research, Inc., Manson, WA).

RESULTS AND DISCUSSION

Synchronous Fluorescence Scanning of Three Bacterial Pathogens. In order to determine which excitation (λ_ex) and emission (λ_em) wavelengths could best detect three bacteria, the synchronous fluorescence scan was chosen. The selection of the wavelength interval (Δλ) between λ_ex and λ_em is one of the most important experimental parameters in employing the synchronous fluorescence technique. Optimization of Δλ was carried out by measuring the synchronous scan spectra at various Δλ. Figure 1 shows the synchronous scan spectra (Fig. 1a) of the E. coli suspension (10^6 cells/mL) and its contour plot (Fig. 1b). The two-dimensional fluorescence excitation and emission contour plot provided a better visualization of the optimum λ_ex and Δλ for the sample. The x-axis is for the λ_ex and the y-axis is for the Δλ. Two main λ_exs were observed at 225 nm and 280 nm and the effective values of Δλ were 110–120 nm and 60–65 nm at λ_ex = 225 nm and 280 nm, respectively. This indicates that the maximum λ_em should be 335–345 nm (λ_em = λ_ex + Δλ) at both λ_exs. The synchronous scan for Salmonella and Campylobacter showed nearly the same results as that for E. coli (data not shown).

Figure 2 shows that indeed the fluorescence emission spectra were recorded following excitation at 225 nm (Fig. 2a) and 280 nm (Fig. 2b) of the three bacterial samples. These spectra are the average of 12 spectra for each culture sample. All show a λ_em between 330 and 350 nm. The intensity of the fluorescence spectra with λ_ex = 225 nm was higher than the intensity with λ_ex = 280 nm, when compared at the same concentration at a...
10 nm slit width. The samples with 10⁷ cells/mL showed the saturated fluorescence at 330–350 nm (intensity > 1000) when \( \lambda_{\text{ex}} = 225 \) nm was used (data not shown). Therefore, the \( \lambda_{\text{ex}} = 225 \) nm spectra were compared at 10⁶ cells/mL and \( \lambda_{\text{ex}} = 280 \) nm at 10⁷ cells/mL. For the fluorescence spectra with \( \lambda_{\text{ex}} = 225 \) nm (Fig. 2a), they were characterized by a maximum located at around 340 nm. The background spectrum (from saline) had no band in this region. It is well known that proteins in bacteria show fluorescence in the 300–400 nm range following excitation at 280–290 nm of their tryptophan residues.27 For the fluorescence spectra with \( \lambda_{\text{ex}} = 280 \) nm (Fig. 2b), they were characterized by a maximum located around 310 nm. The peak that appears at 310 nm is due to the Raman scattering of the water.

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ing was applied to the fluorescence spectra to enable differentiation among the three bacteria. The fluorescence spectra of the three bacteria (10^7 cells/mL) collected with λ_{ex} = 280 nm were truncated to a range of 300–450 nm and preprocessed with multiplicative scatter correction followed by mean centering prior to analysis. For each bacterium, 24 fluorescence spectra were collected from duplicate scanning of 12 samples, giving 72 spectra for three bacteria. The spectral data of each bacterium were divided into two groups of calibration set and validation set by splitting the data 2:1, two for calibration and one for validation, giving 16 spectra for calibration and 8 spectra for validation. A PCA model was developed using the calibration set and then applied to the validation set to evaluate the model.

Figure 5 shows the PCA score plots for the three bacteria. The black triangles represent the calibration set and the white circles represent the validation set. The first two PCs (PC1 and PC2) accounted for over 99% of the variation of x-variables (spectral). Three groups corresponding to the three bacteria were clearly separated from each other. The validation samples were all located in their respective groups. The PCA results demonstrated a successful classification between *E. coli*, *Salmonella*, and *Campylobacter*. The reproducibility of the method was proved by the tight clustering of the bacterial samples collected from cultures grown on subsequent days and also from replicated sampling on the same day.

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The PCA results were expanded using varying levels of all three bacteria as shown in Fig. 6. Each bacteria sample was prepared at four different levels (10^7, 5 × 10^6, 2 × 10^6, and 10^6 cells/mL). The emission spectra in the range of 300–450 nm were used for this PCA processing. The ellipses were added manually to emphasize the division between different levels. Each ellipse contains three bacteria with equal concentration levels. The highest-level group was placed on the left of the plot, while the lowest-level group is on the right. The differentiation by concentration of bacteria was successful, explaining over 98% of the variation of x-variables using one PC.

These results show that fluorescence spectroscopy combined with PCA may be a useful technique to detect and differentiate three bacteria, *E. coli*, *Salmonella*, and *Campylobacter*, according to their genus and levels.

**CONCLUSION**

Our results revealed the potential of fluorescence spectroscopy in combination with multivariate analysis for detection and differentiation of *E. coli*, *Salmonella*, and *Campylobacter*. The fluorescence synchronous scan resulted in two maximum excitation wavelengths at 225 nm and 280 nm with one maximum emission wavelength at 345 nm for all three bacteria. The detection limit was approximately 10^3 cells/mL for each bacterial sample, when using the excitation of 225 nm. PCA proved to be a valuable technique to differentiate the three bacteria by their genus as well as their levels. This method,
based on intrinsic fluorescence, is rapid, reproducible, and requires minimum sample preparation. Further research will be conducted on the practical application of this methodology.

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