STUDYING OF THE BACTERIAL GROWTH PHASES USING FOURIER TRANSFORM INFRARED SPECTROSCOPY AND MULTIVARIATE ANALYSIS

HAMZAH M. AL-QADIRI1,7, NIVIN I. AL-ALAMI2, MENGSHI LIN3, MURAD AL-HOLY4, ANNA G. CAVINATO5 and BARBARA A. RASCO6

1Department of Nutrition and Food Technology
Faculty of Agriculture, The University of Jordan
Amman 11942, Jordan

2Water and Environment Research and Study Center
The University of Jordan
Amman 11942, Jordan

3Food Science Program
University of Missouri
Columbia, MO 65211-5160

4Department of Clinical Nutrition and Dietetics
Faculty of Allied Health Sciences, Hashemite University
Zarqa, Jordan

5Chemistry and Biochemistry Program
Eastern Oregon University
One University Blvd., La Grande, OR 97850-2899

6Department of Food Science and Human Nutrition
Box 646376, Washington State University
Pullman, WA 99164-6376

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ABSTRACT

This study examined the potential of Fourier transform infrared (FT-IR) absorbance spectroscopy to detect biochemical changes in bacterial cells that occur during bacterial growth phases in batch culture. Two bacterial strains, Escherichia coli ATCC 25922 and Listeria innocua ATCC 51742 were cultured in brain heart infusion (BHI) broth and incubated at 37°C and cells recovered

7Corresponding author. TEL: (+962-6)-5355000, ext. 22422; FAX: (+962-6) 5355577; EMAIL: h.qadiri@ju.edu.jo

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at: the lag phase (3 h), the log phase (7 h), the stationary phase (24 h), and the
death phase (10 days) from incubation in BHI (n = 2).

Major variations in the biochemical structure of bacterial cells during
growth were observed. Over the range of 1800–900 cm\(^{-1}\), loadings 1 (principal component [PC] 1) and 2 (PC2) accounted for 88% of the total variability (76% and 12%, respectively) for \(E. \) coli cells, and 80% (72% and 8%, respectively) for \(L. \) innocua cells. Changes were attributable to cellular structure of bacterial cells (the variation in nucleic acids, proteins, lipids and polysaccharides) that occur during the growth phases.

**PRACTICAL APPLICATIONS**

The focus of this study was to examine Fourier transform infrared (FT-IR) spectral features of bacterial cells at different growth stages and to determine if variations in composition and distribution of the biochemical components of cells during growth phases can be distinguished using this spectroscopic technique. This could explain the primary functional principle behind detection and discrimination of bacteria by FT-IR spectroscopy.

**INTRODUCTION**

The normal bacterial growth curve has four stages: lag phase, log (exponential) phase, stationary phase, and death phase (Atlas 1988). These phases characterize typical bacterial growth in batch culture where a complex or a defined liquid nutrient medium is used. In this closed environment, the required nutrients become exhausted and the metabolic products start to accumulate due to the bacterial growth (Akerlund *et al.* 1995). Lag, log and stationary phases are characterized by distinct biochemical reactions for synthesis of cellular components necessary for cell growth and division.

During the lag phase bacteria show no growth – although cells increase in size – but begin to prepare for reproduction such synthesis of DNA and cellular enzymes (Garbutt 1997). During the log phase the bacterial biomass increases linearly with time, with the number of bacterial cells doubling with every unit of time. This phase is defined as the balanced growth stage because the average composition of the cells remains constant with bacterial culture properties (i.e., protein and DNA) increasing at the same rate (Akerlund *et al.* 1995). When the stationary phase is reached, there is no longer a net increase in viable bacterial cell numbers and cellular metabolic activity is decreased meaning that the growth rate is equal to the death rate (Akerlund *et al.* 1995). During the transition between log and stationary phase, cellular components are syn-
thesized at unequal rates (unbalanced growth); therefore, the biochemical composition of cells in the stationary phase is different from that in the log phase (Atlas 1988). Bacterial growth is inhibited during the stationary phase since the essential nutrients required for bacterial growth are exhausted and metabolic inhibitory byproducts accumulate. Eventually, death phase is reached where bacterial cells are broken down (cell lysis) due to the additional accumulation of inhibitory byproducts, depletion of cellular energy, and pH changes (Garbutt 1997).

Studying the changes in biochemical composition, specifically the relative amounts and changes in the distribution of various cellular components such as DNA, proteins, fatty acids and polysaccharides during bacterial growth could improve our understanding of microbial physiology as well as the behavior of different bacterial strains in various batch culture systems.

FT-IR spectroscopy is a new technique for studying the biochemical structure of microorganisms. It is used to identify bacteria and yeasts (Helm et al. 1991; Beattie et al. 1998; Rodriguez-Saona et al. 2001; Wenning et al. 2002) and to discriminate foodborne pathogens (Kummerle et al. 1998; Choo-Smith et al. 2001; Mossoba et al. 2003; Al-Holy et al. 2006; Garip et al. 2007). Distinctive spectral “biochemical fingerprints” can be used to differentiate microbial strains and to ascertain how the cellular composition of microbial cells are different (Goodacre et al. 1996; Kansiz et al. 1999; Lin et al. 2004; Winder et al. 2004; Al-Qadiri et al. 2006; Garip et al. 2007). FT-IR spectra between 4000–400 cm\(^{-1}\) include stretching vibrations and deformation of C–H, C=O, N–H, P=O, and C–O–C and which reflect the differences in the biochemical composition of microbial cells i.e., DNA, proteins, polysaccharides and fatty acids (Hong et al. 1999; Kansiz et al. 1999; Oberreuter et al. 2002; Winder et al. 2004). The main objective of this work was to study FT-IR spectral features of bacterial cells at different growth stages and to determine if variations in composition and distribution of the biochemical components of cells during growth phases can be distinguished using this spectroscopic technique.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

Bacterial cells used in this study were obtained form the culture collection in the Department of Food Science and Human Nutrition, Washington State University. Two bacterial strains were studied: *Escherichia coli* ATCC 25922 as Gram-negative, and *Listeria innocua* ATCC 51742 as Gram-positive. Both strains were activated first by transferring bacterial inoculum from refrig-
erated slant to tryptic soy agar (TSA) (Difco, Sparks, MD) followed by incubation of cultured plates at 37°C for 24 h. A well-isolated single colony of each strain was then inoculated into 100 mL of brain heart infusion (BHI) broth (Bacto, Sparks, MD) and strains were grown aerobically at 37°C.

Four treatments of both strains were studied in duplicate \( (n = 2) \). The first treatment (lag phase) was examined after 3 h in BHI at 37°C, the second treatment (log phase) after 7 h, the third treatment (stationary phase) after 24 h, and the fourth treatment (death phase) after 10 days. The bacterial count at each treatment time was determined and the limiting factor for these experiments was the cell numbers at lag phase treatment as the cell numbers were relatively low. To eliminate the effect of the different bacterial counts on spectral measurements, the cell concentration present at lag phase was used for all treatments. To determine the number of bacterial cells during the lag phase, 1 mL from the BHI broth was taken after 3 h from incubation and appropriate serial dilutions were made (using sterile 0.1% peptone; Bacto). Samples were enumerated (in duplicate) by the standard spread plate technique on TSA, plates were then incubated at 37°C for 48 h and bacterial count was recorded as log viable colony-forming unit (cfu)/mL. This procedure was performed after 7 h, 24 h and 10 days from incubation to determine the bacterial count of the other phases. Accordingly, appropriate dilutions were made for log, stationary and death phases using sterile 0.9% saline solutions to give the same bacterial count as lag phase before spectral measurements were obtained.

For the lag phase treatment, 50 mL broth of each strain was transferred after 3 h from incubation to a 50-mL sterile centrifuge tube. The tubes were then centrifuged at room temperature for 15 min at 4000 rpm (CENTRA CL-2 Model 120 Thermo IEC; Thermo IEC, San Jose, CA) to harvest bacterial cells. To reduce the effect of media components and bacterial metabolites, the resultant pellets were resuspended in 10 mL sterile 0.9% saline solutions and centrifuged again; this step was repeated twice. The supernatants were removed and the remaining wet pellets were resuspended in 500 μL sterile 0.9% saline solution and vigorously mixed to obtain a homogeneous cell distribution. A 300 μL of each bacterial suspension was uniformly applied to a 0.2-μm pore size aluminum oxide membrane filter (25 mm OD; Anodisc, Whatman, Inc., Clifton, NJ). Under aseptic conditions, the filters were then air-dried at room temperature (22°C) for 30 min under a laminar flow hood. This procedure yielded a filter with a relatively uniform coating of dried bacterial cells. The Anodisc membrane filter was used because it does not contribute significantly to the bacterial spectra and it gives a smooth and a flat surface for bacterial cell adhesion (Rodriguez-Saona et al. 2001; Lin et al. 2004; Rodriguez-Saona et al. 2004). Samples from the log, the stationary and the death phases received similar treatment except that an initial volume of 10 mL broth was used.
FT-IR Spectroscopy and Measurement

FT-IR bacterial spectra were collected using Thermo Nicolet Avatar 360 FT-IR spectrometer (Thermo Electron, Inc., San Jose, CA). The dried Anodisc membrane filters (covered with a homogeneous and dried smooth layer of examined bacteria) were positioned in direct contact with an infrared attenuated total reflection zinc selenide crystal (Schmitt and Flemming 1998; Rodriguez-Saona et al. 2001). FT-IR spectra were recorded from 4000–600 cm\(^{-1}\) at resolution of 2 cm\(^{-1}\). Thirty spectra were acquired for each sample treatment (growth phase) at room temperature (each spectrum composed of an average of 128 separate scans). FT-IR spectra were mean centered and baseline corrected.

Multivariate Statistical Analysis

The collected FT-IR bacterial spectra were analyzed using OMNIC (Thermo Electron, Inc.) and Delight version 3.2.1 (Textron Systems, Wilmington, MA) software. To reduce the overlapping between relatively similar bacterial spectra, data preprocessing algorithms were carried out before data analysis. Data preprocessing included spectral binning by 2 cm\(^{-1}\) and smoothing with a Gaussian function of 15 cm\(^{-1}\), this followed by a second derivative transformation, 15 cm\(^{-1}\) gap (Huang et al. 2001; Huang et al. 2002; Lin et al. 2003).

The multivariate statistical analysis techniques of principal component analysis (PCA) and soft independent modeling of class analogy (SIMCA) were used to statistically analyze the obtained spectral data (Kansiz et al. 1999; Lin et al. 2003; Oust et al. 2004; Rodriguez-Saona et al. 2004). PCA was used to show if there is a natural clustering in the spectral data through capturing the related variations among bacterial spectra (Goodacre et al. 1998; Rodriguez-Saona et al. 2001; Nilsen et al. 2002). It was also performed to obtain graphical representations of similarities and differences from infrared spectral data (Martens and Naes 1989; Goodacre et al. 1998; Kansiz et al. 1999; Rodriguez-Saona et al. 2001). The first principal component (PC1) characterizes the maximum percentage of bacterial spectral variation followed by the second PC (PC2) that describes the second most important factor of the remaining spectral variation, and so on (Rodriguez-Saona et al. 2004). SIMCA was mainly used to classify samples according to their analogy to the training sample treatments (Hampton et al. 2001–2002).

RESULTS AND DISCUSSION

Table 1 shows the average of viable bacterial counts (log\(_{10}\) cfu/ml) for E. coli and L. innocua strains at the different growth phases after incubation.
on TSA at 37°C for 48 h. As shown from Table 1, the bacterial count during growth was different from phase to phase. Because there is a variation in composition and distribution of bacterial biochemical structure including cell wall and cell membrane structural lipids (phospholipids and lipopolysaccharides), cellular proteins and enzymes, nucleic acids, and polysaccharides this should be reflected in spectral features at each growth stage.

Conventional methods to determine bacterial cell numbers during different growth phases (i.e., standard plate count and optical density) cannot describe biochemical changes that occur throughout the growth process. However, FT-IR spectroscopy was used to characterize these changes through spectral features analysis of various functional chemical groups and polar bonds that reflect the biochemical composition of the cellular constituents.

Figs. 1 and 2 show the representative FT-IR spectra (4000–600 cm\(^{-1}\)) of \textit{E. coli} and \textit{L. innocua} during the four growth phases. In region I (Figs. 1 and 2), major spectral variations were observed in amide I and amide II due possibly to C=O stretching vibrations (~1650 cm\(^{-1}\)) and N–H deformation or bending (~1540 cm\(^{-1}\)) of amides associated with proteins, respectively (Kansiz \textit{et al.} 1999; Choo-Smith \textit{et al.} 2001; Filip and Hermann 2001; Lin \textit{et al.} 2004).

Region II, commonly called the fingerprint region (1300–900 cm\(^{-1}\)), contains vibrational features of cellular proteins (including enzymes), nucleic acids, and cell membrane and cell wall components (Goodacre \textit{et al.} 1996). Variations between experimental treatments in this region could be associated with P=O asymmetric and symmetric stretches of the phosphodiester backbone of nucleic acids at ~1242 and ~1080 cm\(^{-1}\) respectively, and C–O–C stretching vibrations of polysaccharides (1200–900 cm\(^{-1}\)) associated with the bacterial cell wall and cell membrane peptidoglycan layer and lipopolysaccharide outer leaflet (Kansiz \textit{et al.} 1999; Choo-Smith \textit{et al.} 2001; Filip and Hermann 2001; Lin \textit{et al.} 2004).

The death phase for both strains had a distinctive spectral pattern. The absorbance was relatively higher compared to the other growth phases and that

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**TABLE 1.**

THE AVERAGE VIABLE BACTERIAL COUNTS (log\(_{10}\) cfu/ml)* of \textit{ESCHERICHIA COLI} AND \textit{LISTERIA INNOCUA} STRAINS AT THE DIFFERENT GROWTH PHASES IN BATCH CULTURE (BHI BROTH AT 37C)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth phase treatment</th>
<th>Lag (3 h)</th>
<th>Log (7 h)</th>
<th>Stationary (24 h)</th>
<th>Death (10 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{E. coli} ATCC 25922</td>
<td></td>
<td>4.30</td>
<td>6.90</td>
<td>9.70</td>
<td>8.30</td>
</tr>
<tr>
<td>\textit{L. innocua} ATCC 51742</td>
<td></td>
<td>4.08</td>
<td>7.08</td>
<td>9.64</td>
<td>8.65</td>
</tr>
</tbody>
</table>

*Bacteria were counted by spread plating on tryptic soy agar.
BHI, brain heart infusion; cfu, colony-forming units.
FIG. 1. REPRESENTATIVE FT-IR SPECTRA OF *ESCHERICHIA COLI* ATCC 25922 DURING GROWTH PHASES
(A) Lag phase. (B) Log phase. (C) Stationary phase. (D) Death phase.

FIG. 2. REPRESENTATIVE FT-IR SPECTRA OF *LISTERIA INNOCUA* ATCC 51742 DURING GROWTH PHASES
(A) Lag phase. (B) Log phase. (C) Stationary phase. (D) Death phase.
might be due to: (1) cell lysis leading to leakage of the cellular materials outside the cytoplasm causing a net increase in absorbance; or (2) the presence of the dead cells on the membrane filter; these could not be enumerated on TSA but at the same time would have contributed to the microbial cell density on the membrane. However, in spite of the higher absorbance during the death phase, the spectral pattern for death phase was also characterized by a relative depletion of major cellular components (particularly in region II) including proteins, nucleic acids and most predominantly polysaccharides (1100–950 cm\(^{-1}\)) due to the destruction of the cell wall and cell membrane. The depletion of the cellular components might be associated to the reduction in the metabolic activity during the death phase.

Spectral binning, smoothing and second derivative transformation were performed before multivariate statistical analysis to: (1) detect more details about bacterial biochemical structure by increasing the number of discriminative features associated with the bacterial spectra (Pedone \textit{et al.} 2003; Sandt \textit{et al.} 2003); (2) reduce spectral pattern overlapping; and (3) characterize and amplify the related variations among bacterial FT-IR spectra since unprocessed spectral patterns look similar (Kansiz \textit{et al.} 1999; Lin \textit{et al.} 2005). Spectral normalization was conducted to balance the differences in absorbance as a prerequisite to PCA and SIMCA analysis (Holt \textit{et al.} 1995; Lin \textit{et al.} 2003).

The composition and distribution of the biochemical components in bacterial cells are different during growth phases and FT-IR spectroscopy provides a method and an indication of which specific biochemical changes occur during different growth phases. The 15-point second order derivatization of region I for both strains is shown in Figs. 3 and 4. After transformation, the spectral variations (1800–1300 cm\(^{-1}\)) among the four growth phases become more distinctive for both strains. Major variations could be observed in the log, the stationary and the death phases which makes these stages obviously distinguishable from each other and from the lag phase because of unique amide I and amide II bands (proteins) in the range of \(~1650\) and \(~1540\) cm\(^{-1}\). However, relatively minor variations could also be observed in the range of \(~1455\) and \(~1398\) cm\(^{-1}\) from CH\(_3\) and CH\(_2\) asymmetric and symmetric deformation of proteins.

Figs. 5 and 6 show the 15-point second derivative transformation of bacterial spectra in the fingerprint region (region II). The major differences might be seen in the P=O asymmetric stretch of the phosphodiester backbone of nucleic acids (\(~1242\) cm\(^{-1}\)), P=O symmetric stretch of the nucleic acid ribose or deoxyribose moieties (\(~1080\) cm\(^{-1}\)) (Kansiz \textit{et al.} 1999; Filip and Hermann 2001), and C–O–C stretching vibration of cell wall and cell membrane polysaccharides (Kansiz \textit{et al.} 1999; Filip and Hermann 2001). There were very clear differences in polysaccharide spectral features between the log
FIG. 3. REPRESENTATIVE SECOND DERIVATIVE TRANSFORMATION SPECTRA (1800–1300 cm\(^{-1}\)) OF *ESCHERICHIA COLI* ATCC 25922 DURING GROWTH PHASES
Lag phase (---), log phase (—), stationary phase (——), and death phase (—-).
1, 2: C=O stretching vibration and N–H deformation of amides associated with proteins (amide I and amide II); 3, 4: \(\text{CH}_3\) AND \(\text{CH}_2\) asymmetric and symmetric deformation of proteins.

FIG. 4. REPRESENTATIVE SECOND DERIVATIVE TRANSFORMATION SPECTRA (1800–1300 cm\(^{-1}\)) OF *LISTERIA INNOCUA* ATCC 51742 DURING GROWTH PHASES
Lag phase (---), log phase (—), stationary phase (——), and death phase (—-).
See caption of Fig. 3 for details.
FIG. 5. REPRESENTATIVE SECOND DERIVATIVE TRANSFORMATION SPECTRA (1300–900 cm\(^{-1}\)) OF *ESCHERICHIA COLI* ATCC 25922 DURING GROWTH PHASES

Lag phase (—), log phase (—), stationary phase (—), and death phase (—).

1: P=O asymmetric stretches of the phosphodiester backbone of nucleic acids; 2: P=O symmetric stretches and C–O–C stretching vibrations of polysaccharides.

FIG. 6. REPRESENTATIVE SECOND DERIVATIVE TRANSFORMATION SPECTRA (1300–900 cm\(^{-1}\)) OF *LISTERIA INNOCUA* ATCC 51742 DURING GROWTH PHASES

Lag phase (—), log phase (—), stationary phase (—), and death phase (—).

See caption of Fig. 5 for details.
and the death phases. While there was a relative depletion in the polysaccharides peak during the death phase, possibly from cell lysis, the polysaccharides content increased during the log phase showing an increase in cell biomass.

PCA and SIMCA statistical analysis techniques were employed to differentiate and classify bacteria by growth phases (Oust et al. 2004; Rodriguez-Saona et al. 2004). PCA captures the related variations among spectral data and clusters groups with presumptively similar spectral properties.

A mean centered PCA was performed on the second derivative FT-IR spectra. Figs. 7 and 8 show the two-dimensional PCA clustering results from FT-IR spectral data for *E. coli* and *L. innocua* strains at different growth phases. For both strains, clear segregation was observed between the different growth phases. Bacterial cells during the lag, the log and the stationary phases were clearly segregated with distinct clustering of these cells from those in the death phase, which indicates that there is a significant difference in the biochemical composition of bacterial cells during the death phase. However, bacterial cells during the lag and the stationary phases were closely clustered together, but were distinct from bacterial cells in the log phase possibly reflecting spectral differences associated with rapid cell growth during the log phase.

To predict which spectral regions provide the most significant contributions to data variation during the growth phases, loadings plots are used to highlight the contribution of each variable (wavenumber) to each principal component (Kansiz et al. 1999; Lin et al. 2005). The first and the second
loadings plot from PCA analysis over the range of 1800–900 cm\(^{-1}\) for \textit{E. coli} and \textit{L. innocua} strains are shown in Figs. 9 and 10, respectively. Large positive or negative loadings might be associated with spectral regions within the original spectra that are responsible for bacterial differentiation (Holt \textit{et al.} 1995). Over the range of 1800-900 cm\(^{-1}\), loadings 1 (PC1) and 2 (PC2) accounted for 88% of the total variability (76% and 12%, respectively) in case of \textit{E. coli} cells, and 80% (72% and 8%, respectively) in case of \textit{L. innocua} cells. Accordingly, spectral variables in the range of 1800–900 cm\(^{-1}\) provided the majority of the total variance in the FT-IR data compared to other variables. The major contribution to the spectral variation were amide I and amide II bands; plus the CH\(_3\) and CH\(_2\) asymmetric and symmetric deformation of proteins, P=O asymmetric and symmetric stretches of the phosphodiester nucleic acid backbone, and polysaccharide C–O–C stretching vibrations providing further indication that FT-IR could detect changes in the cellular structure of bacterial cells that occur during growth and death.

Sample classification was performed using SIMCA analysis by comparing spectral features of each growth phase with models constructed from spectra from the remaining treatments. Test spectra were assigned to a class based upon the degree of analogy. Table 2 shows SIMCA classification results for each sample treatment (growth phase) for \textit{E. coli} and \textit{L. innocua} strains compared to the other test treatments.
FIG. 9. LOADINGS PLOT OF THE FIRST AND THE SECOND PRINCIPAL COMPONENTS OBTAINED FROM PRINCIPAL COMPONENTS ANALYSIS OF *ESCHERICHIA COLI* ATCC 25922 FT-IR SPECTRA

FIG. 10. LOADINGS PLOT OF THE FIRST AND THE SECOND PRINCIPAL COMPONENTS OBTAINED FROM PRINCIPAL COMPONENTS ANALYSIS OF *LISTERIA INNOCUA* ATCC 51742 FT-IR SPECTRA
As shown from Table 2, bacterial samples during the log phase and the stationary phase were correctly classified for both strains (90% or greater). However, differentiation of cells in the lag and the death phases were less reliable, particularly for *L. innocua*.

**CONCLUSIONS**

FT-IR spectroscopy can be used to determine biochemical changes that occur during bacterial cell growth and to classify and discriminate bacterial cells during the different phases. Differences in Gram-negative and Gram-positive cells are clearly distinguishable. FT-IR spectral features for detection and discrimination of different bacterial strains and between growth phases are primarily in the amide I (\(\sim 1650 \text{ cm}^{-1}\)) and amide II region (\(\sim 1540 \text{ cm}^{-1}\)) that associated with proteins, as well as nucleic acid and polysaccharide moieties in the fingerprint region (1300–900 cm\(^{-1}\)).

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