Discrimination between Bacillus and Alicyclobacillus Isolates in Apple Juice by Fourier Transform Infrared Spectroscopy and Multivariate Analysis

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Abstract: Alicyclobacillus is a causative agent of spoilage in pasteurized and heat-treated apple juice products. Differentiating between this genus and the closely related Bacillus is crucially important. In this study, Fourier transform infrared spectroscopy (FT-IR) was used to identify and discriminate between 4 Alicyclobacillus strains and 4 Bacillus isolates inoculated individually into apple juice. Loading plots over the range of 1350 and 1700 cm⁻¹ reflected the most distinctive biochemical features of Bacillus and Alicyclobacillus. Multivariate statistical methods (for example, principal component analysis and soft independent modeling of class analogy) were used to analyze the spectral data. Distinctive separation of spectral samples was observed. This study demonstrates that FT-IR spectroscopy in combination with multivariate analysis could serve as a rapid and effective tool for fruit juice industry to differentiate between Bacillus and Alicyclobacillus and to distinguish between species belonging to these 2 genera.

Keywords: Alicyclobacillus, Bacillus, FT-IR, PCA, spectroscopy

Practical Application: This research provides further evidence that Fourier transform infrared spectroscopy spectroscopy can be used rapidly and effectively to detect the spoilage of apple juice caused by Alicyclobacillus strains and to discriminate between Bacillus and Alicyclobacillus strains in apple juice.

Introduction

Alicyclobacillus is a spore-forming acidophilic and thermophilic bacterium that can potentially cause spoilage in acidic beverages (Silva and others 1999; Lee and others 2002). This bacterium was previously considered as a part of the Bacillus genus but was then reclassified as a new genus (Alicyclobacillus) using the 16S rRNA comparative sequence analysis (Wisotzkey and others 1992; Chang and Kang 2004). Alicyclobacillus spp. are nonpathogenic Gram positive, rod-shape, and spore-forming bacteria. Alicyclobacillus spp. have been linked to spoilage of acidic beverages with a characteristic medicinal off-odor in commercially pasteurized apple juice (Chang and Kang 2004). This microorganism is considered as one of the most important target spoilage organisms in acidic foods because Alicyclobacillus spp. can cause off-flavor in commercial acidic beverages because they form heat-resistant spores that can grow at low pH (Vercaemmen and others 2012). Spores of B. coagulans are able to germinate and grow at pH values between 3.7 and 4.5 pH and are naturally present in fruit juices such as apple and orange juices (Mallidis and others 1990; Daryaei and Balasubramaniam 2013). Furthermore, it was reported that Bacillus licheniformis and Bacillus subtilis can grow in acidic and acidified foods such as tomato juice and acidified carrot juice (Rodriguez and others 1993; Tola and Ramaswamy 2014). Therefore, it is important to discriminate between Bacillus and Alicyclobacillus in acidic beverages such as apple juice. In addition, it is important to discriminate between different Alicyclobacillus spp. because not all Alicyclobacillus spp. are capable of producing guaiacol, the substance that gives rise to an objectionable off-odor in acidic beverages.

Currently, detection of Alicyclobacillus is achieved by several methods, including molecular methods such as PCR or 16S rRNA gene sequence analysis (Yamazaki and others 1997; Murakami and others 1998; Cerny and others 2000), and the combination of membrane filtration with optimized recovery media (Chang and Kang 2004). However, these methods are generally labor intensive, time-consuming, and require skilled personnel to conduct product testing. Furthermore, detection results are always contingent upon different isolation protocols. Chang and Kang (2004) reported that many products containing Alicyclobacillus remained undetected and resulted in spoilage reported by consumers. Therefore, it will be prudent to develop a rapid and sensitive method to detect Alicyclobacillus spp. in apple juice and to differentiate it from its closely related genus, Bacillus; because some Bacillus spp. such as B. megaterium and B. subtilis are also guaiacol producers as Alicyclobacillus (Smit and others 2011). Fourier transform infrared spectroscopy (FT-IR) has shown capacity to provide a rapid, nondestructive analysis to generate
relevant information on microbial classification and identification (Alvares-Ordonez and others 2011). FT-IR needs minimum sample preparation and only takes a few seconds to collect a full spectrum. Alongside, FT-IR can provide insight regarding different components in cell wall and cytoplasm, such as proteins and peptides, polysaccharides, peptidoglycan (murein), and phospholipids. The capability of the FT-IR technique to differentiate between microorganisms involves revealing differences in the molecular vibrations of the examined chemical species in the infrared (IR) region. Hence, subtle differences in the chemical composition of microbial cell contribute to a typical and unique IR, spectral fingerprint (Al-Holy and others 2006). Based on its capacity, the FT-IR technique has been extensively studied to identify and differentiate between many microbes of a particular significance to food. It has been elucidated that FT-IR spectroscopy in combination with multivariate statistical analysis techniques was successfully used to discriminate between several spp. of Bacillus such as Bacillus cereus, Bacillus mycoides, and Bacillus thuringiensis (Beattie and others 1998). In addition, FT-IR technique was successfully used to differentiate between the probiotic B. cereus strains and other wild types such as B. cereus, B. thuringiensis, and B. mycoides (Mietke and others 2010). Nonetheless, the Bacillus strains used in the aforementioned study were tested in a pure culture but not in a real food system.

In addition, FT-IR technique has been used to identify and classify many microorganisms including yeast, cyanobacterial strains, lactic acid bacteria, Bradyrhizobium japonicum, Listeria spp., Staphylococcus spp., Clostridium spp., and Escherichia coli O157:H7 (Zeroual and others 1994; Holt and others 1995; Lefier and others 1997; Goodacre and others 1998; Kümmerle and others 1999; Oberreuter and others 2000; Al-Holy and others 2006). FT-IR analysis was also capable of discriminating and identifying pathogenic strains of Listeria monocytogenes (Rebuffo-Scheer and others 2007). Schawe and others (2011) also demonstrated that FT-IR spectroscopy can be used to quantify bacterial species individually even when bacterial cells are present in a mixed culture and at different growth phases. In this study, FT-IR spectroscopy was investigated, since it is a rapid and easy-to-use technique that requires minimal sample preparation. Therefore, the aim of this study was to evaluate the potential of using FT-IR spectroscopy to discriminate between Bacillus spp. and Alicyclobacillus spp. inoculated into apple juice. A 2nd objective was to investigate the capability of this technique to differentiate between species that belong to each of Bacillus and Alicyclobacillus, respectively.

**Figure 1**—Representative FT-IR spectra of Bacillus spp. (A) and Alicyclobacillus spp. (B) recovered from inoculated apple juice.
Materials and Methods

Preparation of bacterial cultures

The bacterial strains used in this study were obtained from the culture collection of the Food Microbiology Lab at Washington State University. Four Bacillus strains (B. cereus ATCC 10876 and ATCC 13061, B. coagulans, and B. subtilis isolate). The latter 2 Bacillus strains were isolated from apple juice. Four Alicyclobacillus isolates were used, A-Gala 2-1, 18-1, 14-2, and C-Fuji 6. All of the Alicyclobacillus isolates are apple juice isolates. The Bacillus isolates were kept on tryptic soy agar (TSA; Difco Laboratories, Detroit, Mich., U.S.A.) slants and were resuscitated aerobically prior to experiment on TSA for 24 h at 37 °C and then transferred to 10 mL of brain heart infusion (BHI; Difco Laboratories) broth. Although, the Alicyclobacillus strains from refrigerated slants were activated by streaking onto potato dextrose agar (PDA, pH 5.6, Beckton, Dickenson, Cockeysville, Md., U.S.A.) at 43 °C for 48 h. A representative colony was then inoculated into 10 mL of BHI and incubated at 37 °C for 48 h. At this point, the cells (approximately 10^6 to 10^7 CFU/mL) were ready for further use.

Apple juice inoculation

Apple juice (pH 3.7; Treetop Inc., Selah, Wash., U.S.A.) was purchased from a local grocery store in the day the experiments conducted. Samples of 50 mL of apple juice were inoculated individually with 1 mL of BHI containing either Bacillus or Alicyclobacillus isolates. Each of Bacillus and Alicyclobacillus was grown in apple juice for 1 wk at 43 °C, then harvested by centrifugation of 50 mL of apple juice at 4000 rpm for 15 min. The obtained pellet was resuspended again into 9 mL of 0.9% saline and centrifuged again as mentioned earlier. The process was repeated twice to remove residual apple juice and bacterial metabolites. The resulting pellet was suspended into 3 mL of 0.9% saline solution and vortexed vigorously to obtain a homogeneous cell distribution. Thereafter, an aliquot of 500 μL of the bacterial suspension was dispensed onto an aluminum oxide membrane filter (0.2 μm pore size, 25 mm OD; Anodisc, Whatman Inc., Clifton, N.J., U.S.A.). Similarly, 500 μL of apple juice was applied onto an Anodisc membrane as a control. The Anodisc filters were subsequently air dried under laminar flow at room temperature for 1 h to allow the formation of a homogeneous dried film of bacterial cells. The experiment was repeated in duplicate. Bacillus counts were determined using a standard spread plating method on TSA. The plates were incubated at 37 °C for 24 h. Alicyclobacillus counts were determined on PDA and the plates were incubated at 43 °C for 48 h.

FT-IR spectroscopy

Thermo Nicolet Avatar 360 FT-IR spectrometer (Thermo Electron Inc., San Jose, Calif., U.S.A.) was used for the FT-IR spectra collections. To collect spectra, the Anodisc membrane filters coated with homogeneous bacterial cells were placed in direct contact with an attenuated total reflection (ATR) zinc selenide (ZnSe) crystal as described by Schmitt and Flemming (1998). Sixty spectra of each sample were acquired at room temperature for each bacterial isolates. FT-IR spectra were recorded in the range of 500–4000 cm^{-1} and the resolution of measurements was set as 4 cm^{-1} with average of 36 scans for each spectral collection.

Multivariate analysis

Data analysis were carried out using OMNIC (Thermo Electron Inc.) and Delight version 3.2.1 (Textron Systems, Wilmington, Mass., U.S.A.) softwares. FT-IR spectral features often look similar and differences between bacterial strains are very subtle. Therefore, some data preprocessing algorithms were employed to analyze the data, such as binning, smoothing, and 2nd derivative transformation (Lin and others 2003). The spectra were smoothed using Gaussian function over 4 cm^{-1} followed by 2nd derivative transformation with a gap value of 12 cm^{-1}. The principal component analysis (PCA) was used to analyze the spectral data. PCA, one of the most commonly used multivariate statistical analysis technique, has been widely employed in the interpretation and extraction of infrared spectral data variance. It reduces a multidimensional data set to its most dominant features, removes the random variation (noise), and retains the principal components (PCs) that capture the related variation (Goodacre and others 1998). PCA analysis shows whether there are natural clusters in the data and describes similarities or differences from

![Figure 2–Principal components analysis (PCA) of Bacillus (BC ATCC 10876, BC ATCC 10361, B. subtilis, and B. coagulans) and Alicyclobacillus (A-Gala 2-1, 18-1, 14-2, and C-Fuji 6) isolated from inoculated apple juice.](image)
multivariate data sets (Martens and Naes 1989). Soft independent modeling of class analogy (SIMCA) was used to assign test samples to a category based upon the analogy of the spectra for the test sample to those in a training set (Lin and others 2005).

Results and Discussion

FT-IR spectroscopy was used in this study to discriminate between isolates of *Bacillus* and *Alicyclobacillus* inoculated into apple juice. *Bacillus* spp. and *Alicyclobacillus* spp. counts ranged between $1.1 \times 10^5$ and $7.8 \times 10^6$ CFU/mL of apple juice. The ability of the FT-IR spectroscopy to differentiate between microorganisms relies on detecting discrepancies in molecular vibrations of the tested chemical species (Al-Holy and others 2006). These differences originate mainly from slight differences present in the bacterial cell wall and cell membrane as well as in the composition of bacterial cytoplasm (Al-Qadiri and others 2006).

Figure 1 shows representative spectra of 4 different isolates of *Bacillus* and other 4 isolates of *Alicyclobacillus*. The FT-IR absorbance spectra exhibited by each bacterial strain are related to the interaction between FT-IR spectra and the generic functional groups found in bacterial cellular constituents. Each bacterial strain elicited a unique absorbance pattern between 500 and 4000 cm$^{-1}$. One of the main compositional differences that distinguishes *Alicyclobacillus* from other *Bacillus* spp. is the presence of $\omega$-alicyclic fatty acids predominantly in their cell membrane (Smit and others 2011). The absorption peaks noticed around 2850 to 3000 cm$^{-1}$ emanate mainly from the CH$_2$ and CH$_3$ stretching vibrations of fatty acid in bacterial cell wall. In the case of *Alicyclobacillus* spp., these vibrations possibly come from the presence of $\omega$-alicyclic fatty acids as a unique and major cell membrane constituent (Lin and others 2005). Although $\omega$-alicyclic fatty acids are characteristic components of *Alicyclobacillus* spp., some *Bacillus* spp. were found to contain $\omega$-alicyclic fatty acids too, albeit in small quantities (Smit and others 2011). Therefore, the presence of $\omega$-alicyclic fatty acids in relatively higher concentrations compared to *Bacillus* spp. may impart certain differences in the FT-IR spectra of the 2 genera.

Additional prominent peaks were noticed at around 1600 and 1700 cm$^{-1}$, most likely originate from protein amide and from...
C = O stretch of fatty acid esters, respectively (Al-Holy and others 2006). The band at 1235 cm⁻¹ is assigned to p = o asymmetric stretching of phosphodiester associated with phospholipid bilayer. Meanwhile, the band at 1080 cm⁻¹ corresponds to nucleic acids (Lu and others 2011). Usually, the chemical composition of bacterial cells determines the FT-IR spectrum (Beattie and others 1998). Although bacterial cells have essentially the same cellular constituents, there are some slight differences in the composition and the distribution of the functional groups that could be unveiled using FT-IR spectroscopy. Based on the visual appearance of the spectra exhibited in Figure 1 alone, the subtle differences between genera and species cannot be uncovered because of the minor compositional differences in their cellular constituents. Hence, data preprocessing algorithms were performed to magnify variations between FT-IR spectra. PCA was used to capture variations among Alicyclobacillus spp. and Bacillus spp. spectra and eliminate noise (Rodriguez-Saona and others 2001). In addition, PCA was used to reveal differences among the 8 related bacterial strains based on the discrepancies in their spectral features and to cluster bacterial cells into groups with potentially similar spectral properties (Lin and others 2005). Figure 2 shows a 2-dimensional projection of the PCA results from mean-centered spectra of the Bacillus and Alicyclobacillus strains investigated in this study. The PCA results show a distinct separation and clustering of each bacterial isolate. The discrimination was not only observed between Bacillus and Alicyclobacillus but also between the different species of the same genera, indicating that FT-IR in combination with multivariate data analysis can be effectively used to discriminate between closely related genera and even among different species of the same genera. The capability of the FT-IR technique in combination with PCA analysis to detect differences among different bacterial types is based upon unique biochemical features of the different bacteria. PCA captured discrepancies among bacterial spectral data and clustered them into visually distinctive groups with presumptively similar spectral properties. Schmitt and Flemming (1998) indicated that FT-IR technique in association with multivariate analysis can reveal variations in the quantity and distribution of bacterial cellular components such as proteins, phospholipids, polysaccharides, peptidoglycans, and nucleic acids, making it possible for the FT-IR to discriminate between closely related bacterial species.

Figure 3 shows the 1st, 2nd, and 3rd loading plot from PCA analysis over the region 1350 to 1700 cm⁻¹. Loading plot underlines the contribution of each variable (wavenumber) to each PC, and highlights the spectral region that provides the major contribution to data variation (Al-Qadiri and others 2006). Variables in the range 1350 to 1700 cm⁻¹ elicited the greatest contribution in the FT-IR spectral data for Bacillus and Alicyclobacillus in apple juice. Loadings 1 to 3 explained 75% of the total variability in the data. Therefore, the variability in the spectra in this particular range (1350 to 1700 cm⁻¹) was the major contributor that discerned the total variance in the FT-IR spectra compared to other variables. The loadings observed in Figure 3 include the following spectral assignments: peaks at approximately 1650 and 1540 correspond to amide I and II, respectively. Peaks at 1455 and 1400 correspond to the asymmetric CH₂ bending mode and to the symmetric vibrations of protein methyl groups, respectively (Maquelin and others 2002; Lin and others 2005).

Figure 4 shows SIMCA classification results of Bacillus spp. (triangles) and Alicyclobacillus spp. (diamonds) in apple juice. SIMCA analysis is based on a PCA model developed for each class in the training set. Treatments are assigned to a class according to their analogy to the training samples. In general, as the percentage of the correctly classified spectra increases, the characteristics of the tested samples show more similarity to the training set. Using SIMCA analysis, it was possible to correctly classify around 78% of the spectra of Bacillus strains and approximately 79% of the spectra of Alicyclobacillus strains. Therefore, this technique could be used to discriminate between Bacillus and Alicyclobacillus isolates. In a study by Rodríguez-Saona and others (2001), SIMCA exhibited a good cluster separation between closely related isolates of Escherichia coli and Bacillus spp. (B. cereus and B. thuringiensis) spiked into apple juice. Lin and others (2005) successfully used SIMCA to correctly classify and differentiate between guaiacol producing and non-guaiacol producing strains of Alicyclobacillus. SIMCA was also employed to compare spectral features of 3 foodborne pathogens (E. coli O157:H7, Campylobacter jejuni, and Pseudomonas aeruginosa) exposed to cold stress injury. SIMCA successfully segregated intact and injured cells of each particular pathogen and differentiated between the 3 pathogens present in low nutrient media (Lu and others 2011).

Conclusions
This study demonstrated the capability of the FT-IR spectroscopy in combination with multivariate analysis techniques to discriminate between phenotypically and compositionally related genera (Bacillus and Alicyclobacillus) in apple juice. In addition, the technique showed a potential to discriminate between closely related species that belong to the Bacillus and Alicyclobacillus genera. This method may serve as a helpful tool to rapidly identify Bacillus and Alicyclobacillus contamination in apple juice industry and therefore may help in detecting and preventing apple juice spoilage.

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References


