Rapid Analysis of Spores and Swiss Cheese Bacterial Cultures by Infrared Microspectroscopy

THESIS

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By

Veena Prabhakar, B.Tech.
Graduate Program in Food Science & Technology

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Thesis Committee:

Dr. Luis E. Rodriguez-Saona, Advisor
Dr. James W. Harper
Dr. Jeff Culbertson
ABSTRACT

Foods we consume are rarely sterile and most foods host a mixture of microorganisms that range from beneficial cultures to pathogenic varieties. In any food industry, microbial analysis forms an integral part of qualitative analysis. Conventional microbial tests usually require several steps of analysis in order to make a positive identification of the microorganism in interest. New methods for rapid analysis of microorganisms are being constantly developed as an alternate to conventional microbial analysis. The objective of this study was to explore the potential of Fourier transform infrared (FTIR) spectroscopy in rapid microbial analysis: classification of Swiss cheese cultures and quantification of Dipicolinic acid release from spores.

For the classification of Swiss cheese cultures, forty four strains of starter and non-starter cultures including *Streptococcus thermophilus*, *Propionibacterium freudenreichii* and *Lactobacillus* spp., previously verified by PFGE, were grown in broth media, centrifuged and the pellets were resuspended in saline solution. The suspension was applied onto hydrophobic grid membrane filters and the dried filters were analyzed using FTIR microscope to obtain the sample spectra. Collected spectra were statistically analyzed by a Soft independent modeling of class analogy (SIMCA) to develop individual classification models for strains of *Streptococcus thermophilus*, *Propionibacterium freudenreichii* and *Lactobacillus* spp. strains. The developed method
allowed for rapid classification of several Swiss cheese starter and non-starter cultures at the strain level. This information provides a detailed overview of the microbiological status, which would enable corrective measures to be taken early in the cheese making process, limiting production of inferior quality cheese and minimizing defects.

For the quantification of Dipicolinic acid (DPA) release from bacterial spores, *Bacillus amyloliquefaciens* Fad 82 cultures grown on two different sporulation media were subjected to pressure-assisted thermal processing (PATP; 600 MPa at 105°C) and high-pressure processing (HPP; 600 MPa at 35°C) for different holding times. Aliquots (1 ml) of the processed samples were centrifuged and the supernatant was analyzed using a fluorometer (reference method) to determine the released DPA concentration. Pellets were dried on a hydrophobic grid membrane filter and analyzed using FTIR microspectroscopy. The collected spectra were correlated with the DPA concentrations obtained from the fluorometer to develop prediction model based on partial least square regression (PLSR). The model could predict the amount of DPA released from the test samples within an error of less than 15 µM regardless of the high pressure processing method or the sporulation media. FTIR spectroscopy provided valuable biochemical information along with quantification of DPA release. The developed method could be effectively utilized as a rapid method to understand the processing resistance of bacterial spores.
DEDICATION

To my parents Dr. T. G. Prabhakar and Vatsala Prabhakar

Thank you for your love and support.
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I would like to thank my parents for being supportive throughout my academic life. I am grateful for their love and encouragement.

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VITA

August 1986 ............................. Born – Tamilnadu, India.

2004 – 2007 ............................... B.Tech. Food and Process Engineering,

                   SRM University, India.

2007 – Present ............................ Graduate Research Associate,

                   The Ohio State University.

FIELDS OF STUDY

Major Field: Food Science and Technology
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1.1. INTRODUCTION

A microorganism is a microscopic form of life found on all non-sterilized matter that can be decomposed (Marriott and Gravani, 2006). Microorganisms like bacteria and virus are ubiquitous and identification of pathogenic and non-pathogenic microorganisms is critical to the food industry (Rösch et al., 2003). Foods we consume are rarely sterile and most foods host a mixture of microorganisms that range from beneficial cultures to pathogenic varieties (Adams and Moss, 2000). Even though the harmless and beneficial microorganisms outnumber the pathogens, food safety calls for thorough microbial analysis. In any food industry, microbial analysis forms an integral part of qualitative analysis.

Effective food microbiological analysis methods are expensive, labor intensive and require long time periods for completion (Gibbs, 1984). Conventional microbial tests usually require several steps of analysis in order to make a positive identification of the microorganism in interest. These steps include: pre-enrichment, selective enrichment, biochemical screening and serological confirmation (Tietjen and Fung, 1995). The tests...
require high level of expertise in molecular biology and the data obtained is complex and
difficult to interpret (Hobson et al., 1996).

New methods for rapid analysis of microorganisms are being constantly
developed as an alternate to conventional microbial analysis. Current research focuses on
development of methods that can rapidly detect low concentrations of pathogens in food.
A number of instruments, like bioluminescence, flow cytometry, have been developed for
rapid detection of microorganisms in food (Van Emon et al., 1995; Wyatt, 1995). These
methods, although very precise, have limited application in the industry because are
tedious and need highly skilled labor. Development of new rapid analysis methods that
are simple and fast could overcome these limitations.

1.2. CONVENTIONAL MICROBIAL ANALYSIS

Microorganisms are important to us because they are added in products valuable
to us. They can also be a threat due to their ability to cause foodborne illnesses. These
two characteristics of microorganisms call for methods to identify them in foods we
consume. Traditional food microbiological analysis generally requires a sequence of
procedures prior to final microscopic analysis. These procedures include preenrichment,
enrichment and selective enrichment (Sperber et al., 2001). Confirming the identity of the
foodborne pathogens include complex molecular subtyping techniques like pulsed field
gel electrophoresis and polymerase chain reaction (Weitzman et al., 2001). The
complexity of these procedures varies depending upon the microorganism in concern.
1.2.1. Analysis of beneficial microorganisms

Beneficial microorganisms are those that do not cause harm when consumed by humans. They are generally used as probiotics, bio-control agents or starter cultures to produce fermented foods and beverages (Giraffa, 2004).

Probiotics are usually present in yogurt products and are known to promote digestive health. These health promoting effects may be enhanced or affected by each individual strain of the multistrain probiotic cultures. Interrelationship of strains of probiotic bacteria may enhance certain probiotic characteristics including metabolic activity. Positive interactions like these are termed as protocooperation and they induce beneficial traits for the added probiotic culture (Timmermana et al., 2004).

In a work done by Huys et al. (2006) it was reported that more than 28% of the commercial cultures intended for human probiotic use were misidentified at the genus or species level, out of which many cases of probiotic product mislabeling originate from the incorporation of incorrectly identified strains. A large number of these discrepancies were considered to be related to the use of methods with limited taxonomic resolution or that are unsuitable for reliable identification up to species level.

Starter cultures, used in fermented products, play a vital role in the final products quality. During cheese ripening, a complex series of reactions takes place when the starter cultures combine with secondary microflora and these reactions lead to the unique flavor and texture of the cheese. Identification of the starter culture is essential to maintain uniform quality of the final product. A wide range of techniques are available
which can be divided into three groups: (1) methods which depend on cultivation followed by phenotypic characterization; (2) methods which depend on cultivation followed by molecular characterization; and (3) methods which depend on molecular characterization only (Beresford et al., 2001).

Conventionally, biochemical methods based on cell morphology are used for identification of starter and non-starter cultures at genus level (Chen et al, 2009). Species level identification of these cultures is performed using various methods like protein fingerprinting, multilocus enzyme electrophoresis, lipid profiling, polymerase chain reaction and ribotyping (Kocaoglu-Vurma et al., 2008).

Although lactic acid bacteria (LAB) identification is carried out through physiological and biochemical properties, like carbohydrate fermentation and certain enzyme activities, only minor differences in phenotypic features is often observed between closely related species. Molecular typing techniques like DNA-DNA – hybridization, polymerase chain reaction (PCR), SDSPAGE of whole-cell proteins and variations of restriction enzyme analysis are applied for LAB identification (Holzapfel et al. 2001; Tannock 2001). Strain level differentiation using genotypic methods are PCR-based methods or variations of restriction enzyme analysis. Pulsed-field gel electrophoresis (PFGE) is regarded as a highly reproducible method applicable to strain-specific differentiation of cultures of *Lactococcus* and *Leuconostoc* species (Kelly et al. 1995; Kelly et al. 1998 Mannu et al. 2000).
1.2.2. Detection of pathogens

Pathogens are defined as microbes that are provided with some virulence factors that render the microbes pathogenic so as to cause disease in a host (Gomes et al., 2006). Pathogens cause cellular damage by establishing in the tissue and the outcome of the damage is either morbidity or mortality. They pose a serious threat as their contamination may result in serious conditions like food intoxication, toxicoinfection and infection (Bhunia, 2007).

Conventional identification of these pathogens is performed by growing them in culture media, isolating them, followed by biochemical and serological identification (Fratamico and Bayles, 2005). Although the conventional identification is considered to be the most reliable method of identification, they are time consuming and laborious (Groody, 1996).

Every year, approximately 76 million cases of foodborne are reported in the United States. The Center for disease control and prevention (CDC) estimates that there are 325,000 hospitalizations and 5,000 deaths related to foodborne diseases each year (CDC, 2005). Foodborne disease is extremely costly and health experts estimate that the yearly cost of all foodborne diseases in the United States is 5 to 6 billion dollars in direct medical expenses and lost productivity (NIAID, 2007). Although the common pathogens include Salmonella spp., Listeria monocytogenes and Staphylococcus aureus, spore forming bacteria like Clostridium botulinum, Clostridium perfringens and Bacillus cereus are also known to cause diseases in humans (FDA, 2009). Some of the pathogens can be
destroyed by proper heat treatments, however bacterial toxins and spore formers resistant to heat and are not inactivated by pasteurization (Table 1.1.).

The bacterial spores of *Bacillus* spp. are metabolically dormant and resistant to many treatments including harsh chemicals, UV radiation and wet and dry heat that are used in killing vegetative bacterial cells (Nicolson et al. 2000; Setlow et al. 2000). In a study conducted by Giffel et al., it was shown that some species of Bacillus species were able to survive at temperatures above 120°C and thus making them able to spoil heat-treated dairy products with a long shelf-life (Giffel et al., 2002). In the 2001 attacks on the United States, *Bacillus anthracis* was used as a bioterrorist agent and it was reported by the CDC that about half of the cases that inhaled anthrax ended up in death (CDC, 2003).

Bacillus spores are found even in acid foods like tomatoes, onions, tomato juice and green peppers and are capable of increasing the pH of these foods, making the growth of pathogenic *Clostridium botulinum* possible (Montenville, 1982; Montenville and Sapers, 1981). A work published by Vaughn et al. claims the isolation of Bacillus spores from canned peaches in sucrose syrup and other foods of low pH ranging from 3.8 to 4 (Vaughn et al., 1952).

Enumeration of bacterial spores from food is generally done by heating the food suspensions and plating serial dilutions on agar media. However, conventional identification often gives underestimated spore population as heat resistance of the spores found on foods differ from those grown on pure cultures (Fritze and Clause, 2003).
Confirmatory tests used to identify spore-forming *Bacillus cereus* is carried out by either testing the colonies for rhizoid growth or appearance of hemolotic area or by using motility test. Protein-toxic crystal stain observed under an oil-immersion microscope gives a strong indication of Bacillus contamination. These tests take about 3 to 4 days to make a positive identification (Lattuada and McClain, 1998).

Identification of *Clostridium perfringens* involves plating serial diluted samples, incubation and colony selection followed by confirmatory procedures. Confirmation testing of these spores involves an initial examination of the plates for contaminant colonies, followed by a second incubation. Colonies from the second incubation are examined for nitrate reduction and carbohydrate fermentation to make a positive identification (McNamara and Lattuada, 1998).

1.2.3. **Need for rapid methods**

Although the conventional methods of microbiological analysis are considered to be the gold standard in its field, the tedious and time-consuming procedures involved in these standard methods call for rapid methods and automation. In microbial analysis with regards to food safety and quality, data should be available for analysis as fast as possible for early corrective measures to be taken (Mossel et al., 1994). Traditional methods for detection and identification of cheese microorganisms have involved labor-intensive and time-consuming biochemical and phenotypic characterization of bacteria and often have been insufficient for monitoring specific strains in complex, mixed-strain microbial consortia. Application of typing techniques such as pulsed field gel
electrophoresis (PFGE) and DNA-fingerprinting can identify bacteria up to the strain level however these methods are tedious and require highly skilled staff which has limited their routine use in industry because of their sophistication. Rapid, reliable and simple techniques for identification, characterization and assessment of the microbial diversity of food microflora could become an alternate to time-consuming conventional analysis methods.

1.3. INFRARED SPECTROSCOPY

1.3.1. Introduction

Infrared (IR) rays are located between the visible light and microwave region of the electromagnetic spectrum (Wehling, 1988). Wavelength of IR region spans from 700 nm to 1,000,000 nm and can be characterized into three distinct regions: near-IR (700 to 2500 nm), mid-IR (2500 to 5 x 104 nm) and far-IR (5 x 104 to 1 x 106 nm). When a molecule’s dipole moment changes due to a normal molecular motion (vibration, rotation, or overtone of these normal vibrations), the molecule absorbs IR radiation in this region of the electromagnetic spectrum (McKelvy et al., 1996).

Absorption of IR radiation by the molecule results in IR spectra due to the vibrational and rotational modes the molecule experiences by absorption of energy (Colthup, 1975). When IR radiation is absorbed by a molecule, it exhibits one of several vibrational or rotational movements depending on the structure and chemical composition of the molecule. IR rays are absorbed at a specific wavelength by each functional group regardless of the molecule that the functional group is in. This correlation between the IR
wavelength and the specific functional group allows the identification of structure of unknown molecules and structural or chemical changes of the molecule to be followed (NG and Simmons, 1999). Also, IR spectra can be collected from materials in either solid, liquid, solution, and vapor phases over a wide range of temperature thus allowing the study of changes in molecular structure of materials in various phases (Putzig et al., 1994).

Infrared spectroscopy was first implemented as an analytical tool in the twentieth century and the scientist that suggested that IR spectroscopy can be used to analyze biological samples was W. W. Coblentz (Coblentz, 1905). However, the technique was not utilized for several decades until the identification of structural properties of intact cells was reported by Naumann, et al. (1984). IR spectroscopy was considered to be time consuming since absorption spectra had to be obtained by scanning the sample using different wavenumbers using a series of prisms to (Norris, 1959). Application of a mathematical transformation operation called fast Fourier transform (FFT) allowed for rapid IR spectroscopic analysis.

1.3.2. FTIR spectroscopy

Fourier transform infrared (FTIR) spectroscopy conceptualized with the invention of interferometer by Michelson in the 1880s (Michelson, 1891; Michelson, 1892). The main component of a FTIR spectrometer is the interferometer (Figure 1.1.) that comprises of three parts: beamsplitter, fixed mirror and moving mirror. Light from a source is split into two by the beamsplitter. One half of the light is directed to the
stationary mirror and gets reflected back to the beam splitter. The other half of the light is
directed to the moving mirror, whose position varies uniformly with time, and gets
reflected back to the beamsplitter. The reflected beams from the two mirrors recombine at
the beamsplitter (Subramanian and Rodriguez-Saona, 2009). Fourier transform (FT) is a
mathematical operation that transforms the time domain into a frequency domain, and
converts the results into a typical IR spectrum (Wehling, 2003). However, FTIR
spectroscopy was not fully utilized until World War II due to the lack of computer and
instrumentation technology.

With the invention of fast Fourier-transformation (FFT) technique in 1965
(Cooley and Tukey, 1965), and application of FFT to IR spectroscopy, resolution of FTIR
saw a significant improvement accompanied by reduced analysis time. Instrumentation
techniques also improved during this period with the introduction of helium-neon lasers,
Improved IR detectors and computation methods which led to the introduction of
commercial FTIR spectrometer. Microsampling was made possible by the introduction of
IR microscope in 1980s. Extensive researches lead to improvement of instrumentation,
computation techniques, sensitivity, speed and detection of the FTIR spectrometers. Now,
FTIR spectroscopy is one of the widely recognized analytical tool and is applied in
various fields of science.

An optical layout of an FTIR spectrometer is shown in Figure 1.2. The major
components of an FTIR spectrometer are the IR radiation source, interferometer, detector,
mirrors to direct light and a time-reference laser. When electric current is passed through
the IR radiation source, light is emitted that travels through the interferometer where the
light beam gets split into two, reflected by stationery and moving mirrors and recombined at the beamsplitter. This recombined beam passes through the sample, produces a signal called interferogram, and is detected by the detector.

FTIR spectroscopy is divided into three areas depending on the wavelength employed as Near, Mid and Far FTIR spectroscopy.

1.3.2.1. FT-NIR spectroscopy

Fourier transform near infrared (FT-NIR) spectroscopy employs NIR wavenumbers range (10000-4000 cm\(^{-1}\)). Spectra collected using FT-NIR technique consists of overtone and combination bands of the fundamental vibration. Overtone peaks are mainly due to the O-H, C-H, S-H and N-H stretching modes and a FT-NIR spectrum is usually complex and difficult to interpret due to overlapping peaks and large baseline variations (Shenk et al., 2001). This technique is utilized by food industries for composition analysis such as rapid measurement of fat, cholesterol, proteins, sugars and moisture.

1.3.2.2. FT-MIR spectroscopy

Fourier transform mid infrared (FT-MIR) spectroscopy involves wavenumbers ranging from 4000-400 cm\(^{-1}\). When molecules absorb energy in this range, they exhibit stretching, twisting, rocking, scissoring and bending motions depending on the structure, bond configuration, etc. This characteristic feature enables composition and structure analysis of the molecules.
FT-MIR spectrum of *Salmonella* Enteritidis and its second derivative with illustrated biochemical composition is shown in Figure 1.3. O-H and N-H stretching vibrations of hydroxyl groups and Amide A of proteins are seen in 4000 to 3100 cm\(^{-1}\). Regions 1700 – 1550 cm\(^{-1}\) and 1310 – 1250 cm\(^{-1}\) represent protein bands. C-H stretching vibrations of –CH\(_3\) and >CH\(_2\) can be seen in region of 3100 and 2800 cm\(^{-1}\). The region from 1200 to 600 cm\(^{-1}\) is called as the “fingerprint region” as it contains signals and important information that are particular to a specific sample and are distinct between each sample. The fingerprint region shows major bands that represent important lipids, proteins, nucleic acids, polysaccharides and phosphate-carrying compounds. List of the functional groups and the wavenumbers that they absorb mid-infrared radiation has been compiled by Coates (2000).

### 1.3.2.3. Microspectrometry

Application of FTIR spectroscopy was extended to microbial analysis with the characterization of bacteria by Naumann (1984). FTIR spectra using microscope provide unique biochemical fingerprints that allows for discrimination between bacterial cells. Hence biochemical information of the bacterial cells can be observed in FTIR spectra with major bands due signals from cellular compounds like lipids, proteins, nucleic acids, polysaccharides and phosphate-carrying compounds (Mariey et al., 2001). Unlike conventional FTIR spectrometers, FTIR Microspectrometry (IRM) requires small amounts of sample for analysis. IRM generally utilizes advanced detectors, like Mercury-Cadmium-Telluride (MCT) that allows for rapid analysis (Katon, 1996).
IRM is now applied in wide range of microbial analysis. Publications are available showing application of IRM to distinguish between bacteria in genus and species level. IRM has also been successfully applied to differentiate between bacteria based on Gram type (Naumann et al., 1991), serological properties (Helm et al., 1991), and temperature preferences (Garip et al., 2007). The application has also extended to discriminating bacterial spores at the strain level (Subramanian et al., 2006). Identification of cellular compounds using IRM, apart from intact cells, have also been reported to identify pathogens (Kim et al., 2006).

1.3.2.4. Attenuated total reflectance

Attenuated total reflectance (ATR) is one of the several sampling techniques (Figure 1.4.) used in FT-MIR spectroscopy. When a beam of IR radiation passes from high to low refractive index medium (Zinc selenide crystal to sample), some amount of light is reflected back to the low refractive index medium. Total internal reflection is a phenomenon that takes place in a particular angle of incidence where all light waves are reflected back.

In ATR technique, the sample is applied over a high / medium refractive index crystal. IR light enters on a end of the crystal from the bottom, undergoes total internal reflection, bounces single or multiple times, and then the light exits from the other end to reach the detector (Figure 1.4 B.). Some amount of the IR energy is absorbed by the sample and translated into a spectrum (Downey, 1998).
1.3.2.5. **Hydrophobic grid membrane filters**

Hydrophobic grid membrane (HGM) filters are special microbial filters with a grid pattern printed on them (Figure 1.5.) to keep bacterial colonies separated from one another and to prevent lateral growth and spreading (Sharpe and Michaud, 1974).

Association of Official Analytical Chemists (AOAC) recognizes this filtration for the enumeration of aerobic bacteria from food and also for detection of *Salmonella* (AOAC, 2000). HGM filtration has been applied to numerous microbial analyses ranging from identification of starter cultures to pathogens. This can be corroborated in detection of Lactococci from cheese starter cultures (Erlandson and Batt, 1997) and isolation of *Listeria monocytogenes* from various foods (Yan et al., 1996). Combination of HGMF with FTIR could be used for rapid identification and detection purposes in microbial analysis.

1.3.2.6. **Advantages and limitations**

FTIR spectroscopy offer several advantages and have some limitations. Speed, simplicity, sensitivity and high signal to noise ratios are often pointed our as important advantages. Need for lower amounts of samples, non-destructive techniques (depending on the application) and less use of chemical solvents makes this method less-expensive (Smith 1996; Halim et al., 2006).

FTIR also poses some limitations. Presence of water in above certain extent can interfere with spectral analysis. The spectral analysis is also influenced by culture
conditions of microorganisms. In some cases, various components overlap at certain spectral regions resulting in complex analysis. FTIR cannot detect monatomic ions, elements, inert gases and diatomic molecules such as N2 and O2 which however is considered advantageous in certain cases as it eliminates the need for vacuum (Naumann, 2000). In spite of these shortcomings, FTIR is used extensively in research as its advantages overcome the limitations.

1.4. CONCLUSIONS

Reliability of FTIR spectroscopy can be verified from its extensive application in food and medical science. Due to technological advances, several improvements have been made in terms of FTIR accessories. Proper sampling methods along with combination techniques allows for high signal to noise ratio and advances in chemometrics have enabled rapid and simple spectral analysis. Other possibilities of expanding FTIR application in food analysis is to be explored.

With this background, FTIR spectroscopy in combination with ATR and HGM filtration was utilized in this research for classification of Swiss cheese cultures and quantification of dipicolinic acid release from bacterial spores.
1.5. REFERENCES


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### Table 1.1. Overview of pathogens found in foods (Source: Snyder, 1994)

<table>
<thead>
<tr>
<th>FOOD</th>
<th>PATHOGENS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FOOD</strong></td>
<td><strong>Ineffective</strong> (Inactivated by pasteurization)</td>
</tr>
<tr>
<td>Meat, Poultry, and Eggs</td>
<td><em>Salmonella</em> spp.</td>
</tr>
<tr>
<td></td>
<td><em>C. jejuni</em></td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> O157:H7</td>
</tr>
<tr>
<td></td>
<td><em>Y. Enterocolitica</em></td>
</tr>
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<td></td>
<td><em>Hepatitis A Virus</em></td>
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<tr>
<td></td>
<td><em>Trichinella spiralis</em></td>
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<td></td>
<td><em>Tapeworms</em></td>
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<tr>
<td>Fin Fish</td>
<td><em>Salmonella</em> spp.</td>
</tr>
<tr>
<td></td>
<td><em>Vibrio</em> spp.</td>
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<td></td>
<td><em>Y. Enterocolitica</em></td>
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<td></td>
<td><em>C. jejuni</em></td>
</tr>
<tr>
<td>Shellfish</td>
<td><em>Salmonella</em> spp.</td>
</tr>
<tr>
<td></td>
<td><em>Vibrio</em> spp.</td>
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<td></td>
<td><em>Shigella</em> spp.</td>
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<tr>
<td></td>
<td><em>Y. Enterocolitica</em></td>
</tr>
<tr>
<td>Fruits, Vegetables</td>
<td><em>Salmonella</em> spp.</td>
</tr>
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<tr>
<td></td>
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<tr>
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<td><em>E. coli</em> spp.</td>
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<td></td>
<td><em>S. aureus</em> (toxin)</td>
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<td></td>
<td><em>C. botulinum</em></td>
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<tr>
<td></td>
<td><em>Bacillus cereus</em></td>
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<tr>
<td></td>
<td><em>Mold (mycotoxins)</em></td>
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<tr>
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<td><em>Y. Enterocolitica</em></td>
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<td><em>Shigella</em> spp.</td>
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<td><em>Norwalk Virus</em></td>
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1.7. FIGURES

Figure 1.1. Schematic diagram of Michelson interferometer (Source: Subramanian and Rodriguez-Saona, 2009).
Figure 1.2. Optical layout of FTIR spectrometer (Source: Subramanian and Rodriguez-Saona, 2009).
Figure 1.3. FT-MIR spectrum of Salmonella Enteritidis (Source: Subramanian and Rodriguez-Saona, 2009).
Figure 1.4. Sampling techniques in FTIR spectroscopy. A – transmission, B – attenuated total reflectance, C – diffuse reflectance in an integrating sphere, and D – specular reflectance. (Source: Subramanian and Rodriguez-Saona, 2009).
Figure 1.5. Hydrophobic grid membranes with bacterial colonies
CHAPTER 2

CLASSIFICATION OF SWISS CHEESE STARTER AND ADJUNCT CULTURES
USING FOURIER TRANSFORM INFRARED MICROSPectroscopy

2.1. ABSTRACT

The acceptability of Swiss cheese largely depends on the flavor profile and the bacterial cultures used in the cheese making have a major influence on the flavor. Strain variations of these cultures have an impact on final quality and hence the price of Swiss cheese. Conventional biochemical methods used to identify the cultures at strain level are time-consuming, expensive and require skilled labor. The objective of this research was focused on building a rapid and cost-effective method for classification of starter cultures at strain level by combination of hydrophobic grid membrane (HGM) filters and Fourier transform infrared (FTIR) spectroscopy. Forty four PFGE verified strains of starter and non-starter cultures including Streptococcus thermophilus, Propionibacterium freudenreichii and Lactobacillus spp. were analyzed. The strains were grown on the respective agar media, transferred to 1 mL of respective broth media and incubated for required conditions. The cultures were centrifuged and the pellets were re-suspended in 10 μL saline solution. Aliquots (2 μL) of the suspended solution was placed onto one grid of the HGM filter, having 6 grids per each strain analyzed. The dried filters were read by
FT-IR microspectroscopy fitted with attenuated total reflectance probe. Collected spectra were statistically analyzed by Soft independent modeling of class analogy (SIMCA) for pattern recognition. SIMCA analysis provided 3D classification models to help visualization of clustering among samples by using score plots and model misclassification tests. Individual classification models were developed for *Streptococcus thermophilus*, *Propionibacterium freudenreichii* and *Lactobacillus* spp. strains. The models showed major discrimination in the spectral region from 1200 to 900 cm\(^{-1}\) associated with signals from various polysaccharides in the cell wall. The developed method allowed for rapid classification of several Swiss cheese starter and non-starter cultures at the strain level. This information provides a detailed overview of the microbiological status, which would enable corrective measures to be taken early in the cheese making process, limiting production of inferior quality cheese and minimizing defects. This method could be an effective tool to identify and monitor activity of cheese and other dairy starter cultures.

### 2.2. INTRODUCTION

Swiss cheese is a hard cheese prepared from pasteurized milk and has holes, or eyes, developed throughout the cheese by microbiological activity (USDA 2001). Although the Swiss cheese making process differs among various countries, the basic steps involved in Swiss cheese making are milk coagulation, removal of whey, acid production, salt addition and ripening. Initial ripening is carried out in warm room for the eye formation to take place by the action of Propionibacteria, and the cheese is moved to cold room to finish ripening process (Beresford et al, 2001). The quality of Swiss cheese
essentially depends on fermentation by lactic acid and propionic acid bacteria, and proteolysis (Steffen et al., 1987). Thermophilic lactic acid bacteria (LAB), such as *Streptococcus thermophilus* and *Lactobacillus helveticus*, and propionic acid bacteria (PAB), mostly *Propionibacterium freudenreichii* spp. *shermanii*, are common starter cultures in Swiss cheese making (Ji et al., 2004).

LAB starter cultures are responsible for homolactic fermentation, converting over 90% lactose into lactic acid (Fox et al., 1993). The produced lactic acid influences Swiss cheese quality in several ways: by preserving milk through pH reduction, facilitating proteolysis and syneresis, and acting as substrate for subsequent propionic acid fermentation (Fox et al., 2000). Upon reaching pH of 5.0 to 5.4, salt addition at 22% inhibits growth LAB, thus arresting lactic acid production, but not propionic acid producing bacteria (Spreer and Mixa, 1998).

Propionic acid bacteria (PAB) are the key microorganisms responsible for the eye formation in Swiss cheese. Propionic acid fermentation utilizes lactate, from lactic acid fermentation, as a substrate to yield acetate, propionate and carbon dioxide (McSweeney and Sousa, 2001). The produced CO$_2$ facilitates in eye formation and PAB also influences the characteristic flavor that distinguishes Swiss cheese from other cheese varieties (Langsurd and Reinbold, 1973).

In addition to the starter cultures, Swiss cheese microflora also comprises of non-starter lactic acid bacteria (NSLAB) (Fox et al., 2000) that usually consists of facultatively heterofermentative lactobacilli (FHL) such as *Lactobacillus casei*, L.
*delbrueckii, L. plantarum* or *L. rhamnosus* (*Weinrichter et al., 2004*). Although NSLAB are generally indigenous to the raw milk or cheese manufacturing facility, intentional addition of selected strains as adjunct cultures has been implemented because of their capability to accelerate proteolysis for cheese flavor development (*Kieronczyk et al., 2003*). It has been reported that FHL adjunct starters may influence the characteristics of Swiss cheeses with respect to various properties (*Weinrichter et al., 2004*) and that cheese manufactured with adjunct lactobacilli showed better flavor intensity (*Lynch et al., 1997*).

Overall, the complex microflora has a major impact on cheese ripening and hence the characteristic features of Swiss cheese. *Lortala and Chapot-Chartier* (*2005*) reported that LAB lysis is an essential parameter to control and accelerate cheese ripening. However, knowledge about genetic similarities and differences are needed for rational culture selection as genetic variations are correlated with cheese making and cheese quality parameters (*Jenkins et al., 2002*). Selection of suitable strains would enable the cheese maker to control or modify flavor development (*Beresford et al, 2001*).

Conventional methods used to characterize the cheese starter cultures are summarized in many publications (*Holzapfel et al., 2001; Tannock, 2001; Jenkins et al., 2002*). These methods include SDS-PAGE of whole-cell proteins, DNA-DNA – hybridization and polymerase chain reaction (PCR). Strain level differentiation is typically done by PCR based methods or restriction enzyme analysis based methods (*Kahala et al., 2008*). Several studies report pulsed-field gel electrophoresis (PFGE) as a highly reproducible method for characterization of starter cultures at the strain level (*Tanskanen et al. 1990; Mannu and Paba 2002; Ward et al. 2004; Lick, 2003; Tilsala-
Fourier transform infrared (FTIR) spectroscopy is an attractive technology for the rapid, inexpensive, sensitive, and high-throughput analysis of microorganisms. FTIR allows for the chemically based discrimination of intact microbial cells and produces complex biochemical fingerprints that are distinct and reproducible for different bacteria. Bacteria have shown highly specific mid-infrared spectral patterns that may be unique for individual strains (Helm et al., 1991; Naumann et al., 1991). FTIR combined with chemometrics have been used for the accurate classification of microorganisms (Wenning et al., 2002; Ngo-Thi et al., 2003; Mossoba et al., 2003). FTIR microspectroscopy combined with hydrophobic grid membrane (HGM) filters was successfully utilized for rapid characterization of *Salmonella* serovars (Mannig et al., 2008). HGM filters have a special printed grid pattern that separate bacterial colonies from one another thus preventing lateral growth, spreading and confluence.

Extending FTIR spectroscopy to classify cheese cultures has not been investigated. The objective of this study was to classify the Swiss cheese starter and non-starter cultures at the strain level using combination of HGM filters, FTIR microspectroscopy and multivariate analysis.
2.3. MATERIALS AND METHODS

2.3.1. Bacterial cultures

Thirteen strains of *Streptococcus thermophilus*, twenty one strains of *Lactobacillus* spp. and ten strains of *Propionibacterium freudenreichii* were utilized for this study. Detailed information of the bacterial cultures can be found in Table 2.1. The cultures were provided by J. T. Parker Chairs Swiss cheese culture collection and all the culture strains were previously identified by PFGE.

*Streptococcus thermophilus* strains were grown in M17 agar containing 0.5% lactose and 0.15% lithium chloride for 2 days at 42°C. Colonies were transferred to M17 broth containing 0.5% lactose and 0.15% lithium chloride and incubated at 42°C for 24h.

Strains of *Lactobacillus* spp. were grown on Rogosa SL agar for 2 days at 37°C in an anaerobe chamber (Forma Scientific, Inc. Marietta, OH) purged with 5% carbon dioxide, 10% hydrogen, 85% nitrogen gas mixture. Colonies were transferred to Lactobacilli MRS Broth and incubated for 24 h at 37°C in an anaerobe chamber.

*Propionibacterium freudenreichii* strains were grown anaerobically in Sodium Lactate Agar for 5-7 days at 30°C. Colonies were transferred to Sodium Lactate Broth and incubated anaerobically for 2 days at 30°C.

2.3.2. Sample preparation

Individual bacterial cultures grown in broth media (1 mL) were centrifuged (8000 rpm; 4°C; 5 min), washed with 0.85% saline solution and the resulting bacterial
pellets were resuspended in 10 µl saline solution. Aliquot (2 µl) of the suspension was applied onto one grid of hydrophobic-grid membrane filters (HGMF, 0.45 µm porous, Neogen Corporation) placed on Iso-Grid filtration unit (Iso-Grid, Neogen Corporation, Lansing, MI). Six grids were utilized for each bacterial strain and the HGMF containing the cultures were dried in a vacuum desiccator for 5 min before analysis.

2.3.3. FTIR microspectroscopy

HGMF containing bacterial cultures were analyzed using an FTIR microscope (UMA 600 series IR microscope interfaced with a FTS Excalibur 3100GX FTIR spectrometer; Varian, Walnut Creek, CA) equipped with a mercury cadmium telluride detector in attenuated total reflectance mode. The microscope was equipped with a motorized x-y stage, a 4x and 16x objective and slide-on attenuated total reflection (ATR) germanium (Ge) objective (Varian 600 UMA, Palo Alto, CA). The instrument had a Permaglow™ (ceramic) mid-infrared source and an extended-range potassium bromide (KBr) beam splitter. FTIR spectra were collected using Varian Resolutions Pro (v4.05, Varian Inc., Palo Alto, CA) in the mid-infrared region (4000-700 cm⁻¹) at a resolution of 8 cm⁻¹. The spectrum of the Ge surface alone was measured as a background reference before sample analysis. The signal to noise ratio was improved by co-adding 128 individual scans and ratioed against the background. A total of six spectra per individual cheese culture strain were collected. The reproducibility of cultures grown on 2 to 5 different days was also examined, resulting in 12-30 spectra per strain to construct the training model.
2.3.4. **Multivariate analysis**

Multivariate analysis of the collected spectral data was performed using the Pirouette® software (Infometrix Inc., Bothel, WA). FTIR spectra were mean-centered, transformed to their second derivative using a 5-point Savitzky-Golay polynomial filter and normalized prior to analysis. Soft independent modeling of class analogy (SIMCA) was utilized to build classification models for the cheese cultures. SIMCA analysis procedure consists of assigning a separate class for each sample and creating principal component analysis model to explain the majority of variation in the data set and grouping samples based on similarities (De Maesschalck et al., 1999). First three principal components are used to project the classes; however more principal components may be used for the actual differentiation. Inter-class distances were calculated using between-class residuals and variable importance was determined by comparing average residual variance of each class to all classes and residual variance of all classes to themselves. Variable importance, also known as discriminating power, was used to define the variables that have a predominant effect on sample classification (Dunn and Wold, 1995).

2.4. **RESULTS AND DISCUSSION**

The identification of Swiss cheese cultures was evaluated by combining the application of hydrophobic grid membrane filters (HGMF) and infrared microspectroscopy to develop direct fingerprinting strategies for starter and non-starter (adventitious bacterial flora) cultures involved in cheese production, in order to help the
cheese-maker to monitor quality cheese and assist to eliminate defects during the ripening process. Conventional approaches of using HGMF involve filtration of inoculated broth media onto the HGM filter and placing the filter directly onto agar media followed by necessary incubation (Sharpe and Michaud 1974; Mannig et al., 2008). In this research work, isolated cheese cultures grown in broth media were centrifuged, resuspended in saline solution and directly applied onto HGM filter, thus eliminating the need of large volume of bacterial cultures, filtration and incubation steps without affecting the inherent advantages of HGMF. The application of HGM filters allowed for the isolation of the bacterial culture in a hydrophobic square which limited horizontal spread and overlap of microcolonies.

Representative raw spectrums of *S. thermophilus*, *L. delbrueckii* and *P. freudenreichii* are shown in Figure 2.1. Transformation of raw bacterial spectra to its second derivative using Savitzky-Golay second derivative algorithm (5-pt gap size) allowed for reduced variability between replications by removing baseline variation and resolving overlapping peaks (Kansiz et al., 2001). Multivariate analysis of spectra resulted in classification models for differentiating between strains of *S. thermophilus*, *Lactobacillus* spp. and *P. freudenreichii*, the main starter and adjunct cultures used in making Swiss cheese (Ji et al., 2004). This protocol allowed for the collection of reproducible infrared absorption spectra directly from biomass of individual colonies isolated with HGM filters thus overcoming the labor-intensive and time-consuming biochemical and phenotypic characterization of bacteria of traditional methods for
detection and identification of cheese microorganisms such as pulsed field gel electrophoresis (PFGE) and DNA-fingerprinting.

2.4.1. Classification of *Streptococcus thermophilus* strains

SIMCA analysis of FTIR spectral data from thirteen *S. thermophilus* cultures showed good clustering of the cultures at strain level (Figure 2.2). This model offered tight clustering of each sample and good separation between samples. SIMCA also offered a 95% probability cloud around each cluster for identification of unknown *S. thermophilus* strains. Visual observation of the model showed close clustering between the strains S 363, A 054, S 838, S 847, S 765, S 731 and S 794 suggesting high similarities between these 7 strains. This observation was confirmed from interclass distance (ICD) values as ICD values between these strains (Table 2.2) were less than or close to 3: generally class distances above 3 are considered to provide good discrimination (Kyalheim and Karstang, 1992). The other strains were well separated and showed ICD values greater than 3. The ICD values show the potential of multivariate analysis to pick subtle differences between the strains.

The pattern recognition model showed good discrimination in the infrared region from 1300 to 800 cm\(^{-1}\), and the wavenumbers having the highest influence on the classification model can be identified in the discriminating power plot (Figure 2.3). Higher values of discriminating power suggest the high influence of the associated wavenumber in classification of the 13 *S. thermophilus* strains and are highlighted in figure 2.2. The major bands / wavenumbers responsible for the classification model were
1146, 1065, 1045 and 991 cm\(^{-1}\). These bands were due to C-O, C-C stretching, C-O-H and C-O-O deformation of the cell components involving polysaccharide components of the cell membrane (Udelhoven et al., 2000; Sahu et al., 2006). Research work by Van der Mei et al. (1996) noted that spectral range from 1300 to 900 cm\(^{-1}\), dominated by bands from phosphate containing compounds and carbohydrates in the cell wall, showed remarkable discriminating power in classifying Streptococcus mitis strains. Work done by Rodrigues et al. (2006) reported strong signals in 1065 cm\(^{-1}\) due to polysaccharides in characterizing biosurfactant produced by S. thermophilus strains. These observations combined with the results from the current results suggest that classification of the S. thermophilus strains are mainly due to the differences in the polysaccharide between the strains.

2.4.2. Classification of Lactobacillus spp. strains

Multivariate analysis of Lactobacillus spp. spectra resulted in a model that classified the strains according to starters and non-starters. The model (Figure 2.4.) showed clear separation between L. helveticus starter cultures from the remaining non-starter strains. However, the non-starter L. delbrueckii strains formed a separate cluster distinct from both the starters and non-starters. The ICD between the three clusters are shown in Table 2.3. It was observed that the L. delbrueckii were clustered closer to the L. helveticus starter cultures (ICD 5.1) than to the other non-starter cultures (ICD 6.7) possibly because L. delbrueckii strains have been reported for being used as starter cultures along with L. helveticus in Swiss type cheeses (Khalid and Marth, 1990).
Four strains of *L. delbrueckii* were included in this research with two strains (C2 and F44) showing closer relatedness to the starters than the remaining two strains. SIMCA analysis provided a model that distinguished the *L. delbrueckii* strains according to their closeness to the starters (Figure 2.5). Strains C2 and F44 clustered closer to the starters (ICD 3.0) than the strains E3 and F1 (ICD 7.6). Marked difference was also observed between the four *L. delbrueckii* strains as there was a good separation between strains C2, F44 and strains E3, F1 (ICD 15.4) indicating phenotypic differences between these strains. It has been shown that FTIR spectroscopy combined with cluster analysis differentiate bacteria based on biochemical properties, rather than taxonomic properties (Naumann, 2000).

SIMCA analysis of the spectral data from *Lactobacillus* strains also developed a model that classified the all strains into individual clusters (Figure 2.6). The clustering pattern showed similarities to the SIMCA model that classified the strains according to starters and non-starters. The six starter *L. helveticus* strains and *L. delbrueckii* C2, F44 strains formed close clusters (marked 8, 12, 16-21, Figure 2.6). *L. delbrueckii* E3 and F1 strains clustered together (marked 10, 11, Figure 2.6) whereas, the remaining non-starters were clustered apart. This data was also supported from the ICD values (Table 2.4) provided by multivariate analysis. ICD values close to 3 were observed in strains that were similar. SIMCA analysis of *Lactobacillus* strains showed the potential of FTIR microspectroscopy combined with HGMF and multivariate analysis as rapid method to classify numerous bacterial samples according to biochemical properties in addition to providing classification at the strain level.
The classification model of *Lactobacillus* spp. strains showed good
discrimination in the infrared region from 1400 to 700 cm\(^{-1}\). The important wavenumbers
that provided the highest discrimination for the classification model can be observed from
the discriminating power plot (Figure 2.7.). The band at 1150 cm\(^{-1}\) had the highest
discrimination and can be associated with signals from carbohydrate C-O stretching of
cell wall carbohydrate components (Nichols et al., 1985). The remaining bands were
related to signals from phosphate containing compounds (1065 cm\(^{-1}\)) and various
oligosaccharides and polysaccharides (1173, 1150, 1042, 1026 and 984 cm\(^{-1}\)) in the cell
wall (Naumann et al., 1995; Naumann et al., 1996). Work done by Luginbuhl et al.
(2006) reported that the infrared region from 1300 to 900 cm\(^{-1}\) was the most effective in
classifying seven species of *Lactobacillus aciophilus* group. It was reported by Amiel et
al. (2001) that the region 1200–900 cm\(^{-1}\), containing signals COC and COP stretching
vibration from polysaccharides, were selective for classification of dairy *Lactobacillus*
spp. at the species and strain levels. All these previous observations, incorporated with
our results, can be utilized to conclude that classification of Swiss cheese *Lactobacillus*
spp. cultures were mainly due to the differences in the polysaccharides and phosphate
containing carbohydrates in the cell wall.

### 2.4.3. Classification of *Propionibacterium freudenreichii* strains

SIMCA analysis of the spectral data from *Propionibacterium freudenreichii*
strains developed a model that classified them into ten classes with good clustering
(Figure 2.8.). The model showed good class separation and tight clustering among the
ten strains with in-between class distances ranging from 2.2 to 13.2. The ICD values of
this model are indicated in Table 2.5. Close clustering was observed within strains P812, P835, P745M1 and P745M2 suggesting similar biochemical characteristics between these strains. Although some of the class distances were lower than 3, presumably due to the subtle differences among the strains, clustering were achieved with major discrimination in the range between 1400 and 700 cm\(^{-1}\).

Figure 2.9. shows the discriminating power plot indicating the important wavenumbers that provided the highest discrimination for the classification model of \(P. freudenreichii\) strains. Infrared region from 900 to 1300 cm\(^{-1}\) resulted in major discrimination of the classification model with important wavenumbers at 1335, 1173, 1153, 1078, 1022 and 962 cm\(^{-1}\). These discriminating bands are similar to those found in Lactobacillus and are presumed to correspond to signals associated C-H bending, C-O stretching, C-O-P stretching and O-H out-of plane bending (Coates, 2000). This research work is the first attempt to classify Propionibacterium cultures using FTIR spectroscopy.

2.5. CONCLUSION

Application of FTIR spectroscopy to classify microorganisms had been successfully implemented by many researchers. This research was aimed at building classification models for several starter and non-starter cultures used in making Swiss cheese. A rapid and simple method was developed to classify over forty Swiss cheese cultures at the strain level using combination of HGM filters and FTIR microspectroscopy. The developed classification model also indicated possibility of biochemical similarities between the strains by forming close clusters. Implementing this
technique would enable the Swiss cheese manufacturer to select the cheese cultures at strain level. This information will enable the cheese-maker to have a detailed overview of the microbiological status of the starter and non-starter cultures, which in turn would enable corrective measures to be taken early in the process, limiting production of inferior quality cheese and minimizing defects. Furthermore, this technique could be extended to classify other cheese and dairy cultures.
2.6. REFERENCES


The authors would like to acknowledge the financial support from the Swiss Cheese Consortium and the J.T. Parker Chair in Dairy Foods.
## 2.8. TABLES

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<th>Lactobacillus spp.</th>
<th>Propionibacterium freudenreichii</th>
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<td>S. thermophilus S 363</td>
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<td>P. freudenreichii P891</td>
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Table 2.1. List of Swiss cheese starter and non-starter culture strains
Table 2.2. Interclass distances between thirteen *S. thermophilus* strains

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*strains of Streptococcus thermophilus*

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* Strains A2, A26, A3, A34, B21 and G2 - L. Casei; strains B4 and B15 - L. fermentum; strains C2, E3, F1 and F44 - L. delbrueckii; strains D56, H1 and H2 - L. rhamnosus; strains S1, S9, S14, S15 and S5 - L. helveticus

Table 2.5. Interclass distances between ten _Propionibacterium freudenreichii_ strains

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* Strains of _Propionibacterium freudenreichii_
2.9. FIGURES

Figure 2.1. Typical FTIR raw spectrums of *S. thermophilus*, *L. delbrueckii* and *P. freudenreichii* bacteria.
Figure 2.2. Soft independent modeling of class analogy plot for thirteen *S. thermophilus* strains

(1) *S. thermophilus* S 363; (2) *S. thermophilus* S 804; (3) *S. thermophilus* A 054; (4) *S. thermophilus* S 341; (5) *S. thermophilus* S 392; (6) *S. thermophilus* S 884; (7) *S. thermophilus* S 869; (8) *S. thermophilus* S 838; (9) *S. thermophilus* S 847; (10) *S. thermophilus* S 728; (11) *S. thermophilus* S 765; (12) *S. thermophilus* S 731; (13) *S. thermophilus* S 794
Figure 2.3. Discriminating power plot for the classification of thirteen *S. thermophilus* strains. Infrared wavenumbers that were different between the thirteen classes and relative extent of difference are shown.
Figure 2.4. Soft independent modeling of class analogy plot for *Lactobacillus* starter and non-starter cultures.
Figure 2.5. Soft independent modeling of class analogy plot for *Lactobacillus* starter, non-starter cultures and *L. delbrueckii* strains
Figure 2.6. Soft independent modeling of class analogy plot for twenty one \textit{Lactobacillus} spp. strains

(1) \textit{L. casei} A2; (2) \textit{L. casei} A26; (3) \textit{L. casei} A3; (4) \textit{L. casei} A34; (5) \textit{L. fermentum} B4; (6) \textit{L. fermentum} B15; (7) \textit{L. casei} B21; (8) \textit{L. delbrueckii} C2; (9) \textit{L. rhamnosus} D56; (10) \textit{L. delbrueckii} E3; (11) \textit{L. delbrueckii} F1; (12) \textit{L. delbrueckii} F44; (13) \textit{L. casei} G2; (14) \textit{L. rhamnosus} H1; (15) \textit{L. rhamnosus} H2; (16) \textit{L. helveticus} S1; (17) \textit{L. helveticus} S2; (18) \textit{L. helveticus} S9; (19) \textit{L. helveticus} S14; (20) \textit{L. helveticus} S15; (21) \textit{L. helveticus} S5
Figure 2.7. Discriminating power plot for the classification of twenty one *Lactobacillus* spp. strains. Infrared wavenumbers that were different between the twenty one classes and relative extent of difference are shown.
(1) *P. freudenreichii* P891; (2) *P. freudenreichii* P812; (3) *P. freudenreichii* P728; (4) *P. freudenreichii* P843; (5) *P. freudenreichii* P835; (6) *P. freudenreichii* P859; (7) *P. freudenreichii* P318; (8) *P. freudenreichii* P745M1; (9) *P. freudenreichii* P745M2; (10) *P. freudenreichii* ATCC 6207

Figure 2.8. Soft independent modeling of class analogy plot for ten *P. freudenreichii* strains
Figure 2.9. Discriminating power plot for the classification of ten *P. freudenreichii* strains. Infrared wavenumbers that were different between the ten classes and relative extent of difference are shown.
CHAPTER 3

QUANTIFICATION OF DPA RELEASE DURING HIGH PRESSURE AND HIGH PRESSURE-TEMPERATURE TREATMENT OF *Bacillus amyloliquefaciens* SPORES GROWN ON TWO DIFFERENT MEDIA USING FOURIER-TRANSFORM INFRARED MICROSCOPY

3.1. ABSTRACT

The release of dipicolinic acid (DPA), which constitutes up to 15% of spore’s dry weight, is believed to play a role in spore inactivation by wet-heat. Rapid methods to quantify DPA release from spores would enable simple and cost-effective study of spore inactivation during food processing. Fourier-transform infrared (FTIR) spectroscopy was used to build a model for rapid prediction of DPA released from treated *Bacillus* spores grown on tryptic soy agar with yeast extract (TSAYE) and nutrient agar with yeast extract (NAYE). *Bacillus amyloliquefaciens* Fad 82 cultures were subjected to pressure-assisted thermal processing (PATP; 600 MPa at 105°C) and high-pressure processing (HPP; 600 MPa at 35°C) for different holding times. Aliquots (1 ml) of the processed samples were centrifuged and the supernatant was analyzed using a fluorometer (reference method) to determine the released DPA concentration. Pellets were dried on a hydrophobic grid membrane filter and analyzed using FTIR microspectroscopy. The collected spectra were correlated with the DPA concentrations obtained from the
fluorometer to develop prediction model based on partial least square regression (PLSR). The models could predict the amount of DPA released from the test samples within an error of less than 15 µM, regardless of the growth media or pressure treatment type. The classification models based to soft independent modeling of class analogy (SIMCA) showed that DPA concentrations were higher in spores grown on NAYE than TSAYE. Furthermore, PATP was found to cause greater DPA release in shorter time compared to HPP treatment. FTIR spectroscopy provided valuable biochemical information along with prediction of DPA release.

3.2. INTRODUCTION

Bacterial endospores (or spores), dormant structures mainly produced by Bacillus and Clostridium, generally demonstrate higher resistance than vegetative cells to variety of treatments, including heat, desiccation, radiation, pressure, and chemicals (Fichtel et al., 2008; Gould, 2006; Rusell 2003; Setlow, 2006). Inactivation of bacterial spores is crucial for sterilization of food and pharmaceutical product. Increasing concerns over biological warfare and bioterrorism (eg. Bacillus anthracis), food safety and food spoilage have fueled the need for safe and rapid methods to detect and inactivate bacterial spores. Inactivation of spores in foods typically requires harsher processing conditions than pathogens, resulting in greater nutrient degradation along with spore inactivation (Matser et al., 2004; Raso and Barbosa-Canovas, 2003). Hence alternative processing technologies to thermal processing such as high pressure processing (HPP), pressure-assisted thermal processing (PATP), ohmic heating, pulsed electric field processing, etc are being sought to achieve food sterilization in a shorter time. Resistance of spores to
pressure does not correlate with their resistance to heat (Nakayama et al., 1996). Due to this reason PATP has received increasing attention as its two-pronged (high pressure and temperature) treatment can be more effective against microorganisms and spores (Ahn et al., 2007; Barbosa-Cánovas and Juliano, 2008). This technology was recently approved by the FDA for processing low acid canned foods. Despite these developments the mechanism of microbial (including spores) inactivation involved in several of the processing methods, including thermal processing which has been used for several decades, is not clearly understood (Gould, 2006).

Several factors, including dipicolinic acid (DPA), small acid soluble proteins, sporulation conditions, presence of minerals, cortex, etc. play a role in the resistance of spores (Russell 2003; Setlow, 2006). Understanding their role in resistance of spores to food processing has been an active area of research for several years. Among the various resistance factors DPA (pyridine-2,6-dicarboxylic acid) has received the greatest attention. DPA, a compound present in bacterial spores, constitutes up to 15% of spore’s dry weight (Murrell, 1969). It is present as DPA molecules or as chelates of divalent cations (eg. Ca$^{2+}$ and Mn$^{2+}$, with calcium chelates being more predominant). Ca-DPA contributes to spore resistance and stability by lowering the core water content (Marquis and Bender, 1985; Russell 2003; Setlow, 2006). Ca-DPA levels in spores can vary with species, strain, and sporulation conditions. Variations in Ca-DPA content might be correlated with heterogeneity in resistance properties of spores in a population. DPA and Ca-DPA are preserved only in intact endospores (Fichtel et al., 2007a) and their
concentrations markedly influence spore resistance. Hence, DPA is regarded as a major biomarker for detection of bacterial spores and determination of spore inactivation.

The role of DPA in spore resistance has been investigated and discussed by several publications (Kort et al., 2005; Paidhungat at al., 2000; Rusell 2003; Setlow, 2006; Slieman and Nicholson, 2001). DPA has also been used a biomarker to detect and quantify bacterial spores (Goodacre et al., 2000; Hindle and Hall, 1999; Pellegrino et al., 2002; Rosen et al., 1997). Studies on inactivation mechanisms and detection of spores require a rapid and reliable tool to quantify DPA. Numerous methods have been reported for extraction of DPA from spores (Pellegrino et al., 2002; Wang and Lin, 2007).

Available analytical methods for quantification of DPA and Ca-DPA are based on liquid chromatography (Fichtel et al., 2007b; Fichtel et al., 2008), gas chromatography (Tabor et al., 1976), Raman spectroscopy (Bell et al., 2005; Huang et al., 2007), mass spectrometry (Beverly et al., 1998), fluorescence (Fichtel et al., 2007a; Hindle and Hall, 1999), colorimetric (Janssen et al., 1958) and UV absorption (White et al., 2004).

Fluorescence method is common and involves complexing DPA with Tb$^{3+}$ to form luminescent complex (terbium dipicolinate). Liquid chromatographic separation followed by fluorescent detection has also been reported (Fichtel et al., 2007b; Fichtel et al., 2008).

All these studies employ laborious and time-consuming techniques like plating, extraction, chromatography and fluorescence. The chemical methods also employ hazardous solvents as an integral part of the technique. The analysis time is further prolonged by the longer incubation periods required for spores.
FTIR spectroscopy is a simple technique that could potentially overcome many of the above limitations and offer a rapid method for DPA quantification. Its applications on characterizing spore components, enumeration of spores and monitoring spore inactivation have been studied by many (Cheung et al., 1999; Goodacre et al., 2000; Perkins et al., 2005; Subramanian et al., 2006; Subramanian et al., 2007). However, the possibility of quantifying DPA release from treated spore samples has not been attempted. The objectives of this research were to 1) determine the amount of DPA released during HPP and PATP treatment by fluorescence method using Terbium and 2) correlate DPA concentrations to FTIR spectrum of samples and develop models to predict the amount DPA released from spore suspensions. The overall goal was to develop a simple, reagent-less and rapid method to quantify the DPA released from spores during HPP and PATP by FTIR spectroscopy.

3.3. MATERIALS AND METHODS

3.3.1. Spore preparation and PATP processing.

*Bacillus amyloliquefaciens* TMW 2.479 Fad 82 cultures, originally isolated from ropy bread, were used in this research. Sporulation and sample preparation were done exactly as detailed by Ratphitagsanti and coworkers (Ratphitagsanti et al., 2009a; Ratphitagsanti et al., 2009b). *Bacillus amyloliquefaciens* TMW 2.479 Fad 82 cultures were grown in trypticase soy broth supplemented with 0.6% yeast extract (TSBYE) with aerobic incubation at 32°C for 24 h. After the second transfer in TSBYE, the cultures were used for spore production. Two batches of spores were produced using two different
media. The first batch was prepared by spread-plating the 100 µl portions of \( B. amyloliquefaciens \) culture on trypticase soy agar supplemented with 0.6% yeast extract (TSAYE) and 10 ppm MnSO\(_4\)H\(_2\)O. The second batch was grown on nutrient agar supplemented with 0.6% yeast extract (NAYE) and 10 ppm MnSO\(_4\)H\(_2\)O. The inoculated plates on TSAYE were aerobically incubated at 32°C for 10-14 days, whereas those of NAYE were incubated for 3-5 days to obtain 95% sporulated population. The sporulation was verified by using a phase-contrast microscopy. The surface of inoculated plates was flooded with 10 ml of cold sterile deionized water and the spores were scraped with disposable plastic spreaders. The spore suspension was washed five times by differential centrifugation that ranged from 2000 to 8000 rpm for 20 min each at 4°C. Spore pellets were re-suspended in sterile deionized water to obtain \(~10^8\) spores/ml for each production. The suspension was sonicated for 10 min following heat treatment at 80°C for 10 min to destroy any remaining vegetative cells. The spore suspension was stored in a refrigerator at 4°C until used.

Procedure described by Ratphitagsanti and coworkers (Ratphitagsanti et al., 2009a; Ratphitagsanti et al., 2009b) was followed for sample preparation and processing. HPP and PATP treatments were carried out using PT1 (Avure Technologies, Kent, WA). The lab scale equipment has approximately 30 s pressure come-up time. Spores (10^8 spores/ml) suspensions were subjected to PATP (600 MPa and 105°C) and HPP (600 MPa and 35°C) for selected holding times as listed later in Table 3.1. In addition \( B. amyloliquefaciens \) spore samples were also treated by PATP with pulsing as described by Ratphitagsanti and coworkers (Ratphitagsanti et al., 2009a; Ratphitagsanti et al., 2009b).
and used as a model validation set (Table 3.3). Samples (1 ml), collected at regular time intervals, were centrifuged (15,200 g and 4°C for 4.5 min) and the supernatant was used for fluorescence assay and the pellet for FT-IR spectroscopy.

### 3.3.2. Fluorometric assay

Fluorescence method using Terbium, reported by Kort and coworkers (Kort et al., 2005) was followed for the determination of DPA. The fluorescence of DPA in spores is based on the emission of terbium (Tb$^{3+}$) ion upon binding with DPA forming (Tb-DPA)$^+$ terbium dipicolinate complex. DPA solutions ranging from 1 to 20 µM were prepared for the standard curve. Terbium reagent was prepared using 100 µM terbium chloride hexahydrate and 20 mM Tris buffer at pH 7.5. The DPA sample and terbium reagent (1:1) were mixed and the fluorescence was monitored using Cary Eclipse Fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA) at 225 nm excitation and 545 nm emission settings. Sample supernatants obtained after PATP treatment and centrifugation supernatants were then analyzed and the amount of DPA released by the sample during PATP treatment were obtained from the standard curve.

### 3.3.3. FTIR spectroscopic analysis

Bacterial spore pellets obtained on centrifugation of PATP-treated samples were resuspended in 10 µl of deionized water. Aliquot (2 µl) of the resulting suspension was applied to every other grid on the hydrophobic-grid membrane (HGM) filters. These filters use a grid pattern, printed in hydrophobic material to separate colonies from one another allowing for orderly arrays of bacterial samples (Figure 3.1. insert). The HGM
filters with samples were dried in a vacuum desiccator for 5 min and left overnight at room temperature in a covered Petri dish. Filter with dried isolated colonies of spores were then placed under the IR microscope (UMA 600, Varian Inc., Palo Alto, CA) equipped with a mercury cadmium telluride detector and scanned in attenuated total reflectance mode in the mid-infrared region (4000-700 cm\(^{-1}\)). Interferograms were collected using the Varian Resolutions Pro (v4.05, Varian Inc., Palo Alto, CA) at a resolution of 8 cm\(^{-1}\) by co-adding 128 individual scans and ratioed against the background to obtain the spectra. Three HGM grids were spotted per sample and 2 spectra were collected from each grid, providing a total of 6 spectra per sample.

### 3.3.4. Multivariate analysis

The collected spectra were imported into Pirouette® (Infometrix Inc., Bothel, WA) software, mean-centered, transformed into their 2\(^{nd}\) derivative and normalized prior to analysis. The correlation between specific fingerprint spectral information of \(B.\ amyloliquefaciens\) Fad 82 spores and DPA concentration obtained from the fluorimetric assay (reference method) was determined using partial least squares regression (PLSR). Fluorometrically determined DPA concentrations were assigned as the dependent variable and the infrared wavenumbers were assigned as the independent variable. Cross-validation with leave-one-out approach was followed to improve model performance. Outliers were eliminated based on outlier diagnostics provided by PLSR. The predictability of the model was validated using five independent unknown samples. The effect of media on DPA release from \(B.\ amyloliquefaciens\) Fad 82 spores during PATP was studied using soft independent modeling of class analogy (SIMCA) analysis. SIMCA
is a classification procedure based on principal component analysis that extracts a small number of latent factors (or principal components; PCs) to explain the majority of variation in the data set and groups samples based on similarities. The chemical differences between spore samples grown on TSAYE and NAYE were investigated by visualizing SIMCA classification clusters and the discriminating power plot, which highlights relative importance of infrared wavenumbers in the SIMCA model.

3.4. RESULTS AND DISCUSSION

3.4.1. FTIR spectra

A novel method of preparing bacterial spores samples using hydrophobic grid membranes for the determination of DPA release and spore inactivation by ATR-FTIR microspectroscopy was developed. Drying spore pellets on HGM filters resulted in well-contained uniform spot of spore sample that allowed for the collection of high-quality spectra with distinct and consistent spectral features. The raw mid-infrared spectrum of Bacillus amyloliquefaciens Fad 82 along with a picture of hydrophobic grid membrane containing isolated bacterial spore samples is shown in Figure 3.1. FTIR spectra reflect the total biochemical composition of the bacterial spore, with bands due to major cellular constituents such as water, lipids, polysaccharides, acids, etc. The region from 4000 to 3100 cm\(^{-1}\) consists of absorbance from O-H and N-H stretching vibrations of hydroxyl groups and Amide A of proteins, respectively. The region between 1800 and 1200 cm\(^{-1}\) has been reported to contain most of signals of interest, including absorption bands of DPA and its compounds, in studying bacterial spores by FTIR spectroscopy.
Some of the important DPA-related IR bands and the associated functional group absorption are listed in Table 3.2. In general, the spectra contain protein bands in the regions 1700 – 1550 cm\(^{-1}\) (amide I and amide II) and 1310 – 1250 cm\(^{-1}\) (amide III). The spectral range 1250 – 800 cm\(^{-1}\) consists of signals from phosphodiesters and carbohydrates. Prior to multivariate analyses the spectra were derivatized (2\(^{nd}\) order) using Savitzky-Golay polynomial filter, to remove baseline shifts and improve resolution and consistency.

### 3.4.2. Quantification of DPA release

The amount of released DPA from the spores of *B. amyloliquefaciens* during HPP and PATP treatment was determined by fluorescence spectroscopy (reference method). A plot of the fluorescent emission at 545 nm vs standard concentrations of DPA showed excellent fit with coefficient of determination (R\(^2\)) >0.99 (data not shown). Using the standard curve the DPA concentrations in treated spore suspensions were determined. Table 3.1. shows the amount of DPA released from *B. amyloliquefaciens* Fad 82 grown on TSAYE and NAYE during HPP and PATP treatments for different holding times. The amount of DPA release thus determined were correlated with the corresponding FTIR spectra of spores and analyzed by partial least squares regression (PLSR) multivariate analysis. The DPA concentrations in spore suspensions determined by fluorescence (dependent variable) were regressed against the independent variables (PLSR factors or linear combinations of wavenumbers) to develop prediction models. The correlation observed between FTIR spectra of spores and DPA release data is shown in Figure 3.2.
The coefficient of correlation (r-Value) was 0.98, indicating a very good linear correlation.

In PLSR analysis of spectroscopic data, the dependent variable (DPA concentration), is related to the wavenumbers through auxiliary variables called PLSR factors or latent variables (Romía and Bernárdez, 2008). PLSR includes as much predictive information as possible in the first few factors, which reduces the effect irrelevant variations in the spectra (noise). The first four PLSR factors explained close to 97% of the variance observed in the dataset, with the first factor alone explaining almost 70%. Figure 3.3. shows the loading spectra for the first PLSR factor in the model. Factor loadings are estimated weights that are applied to the variables while fitting the bilinear relationship between independent variables (DPA concentration) and dependent variables (wavenumbers). The loadings on the PLSR factors provide information on the wavenumbers that influence the PLSR model by loading either high or low. All of the wavenumbers that loaded high in factor 1 belonged to absorption bands of DPA or Ca-DPA, listed in Table 3.2. (1616, 1570, 1440, 1412, 1378, and 1280 cm⁻¹). This signifies high reliability of the developed PLSR model as almost 70% of the variance used to quantify DPA release are based on the compounds of interest (DPA and Ca-DPA).

Validation of the PLSR models was done using 5 independent test samples that were subjected to PATP-pulsing treatment. The amount DPA released (in μM) during treatment, determined by fluorescence and FTIR microspectroscopy are summarized in Table 3.3. The standard error of prediction for predicting DPA release from the five test samples was 15 μM. It should be noted that any variations in the reference Fluorometric
method will adversely influence the predictive capability of the developed model. This data clearly shows that there is potential for this technique to rapidly predict the amount of DPA released from bacterial spores during food processing/sterilization operations.

3.4.3. Effect of growth media

As evident from Table 3.1., the growth media influenced the amount of DPA release during HPP or PATP treatment. *Bacillus amyloliquefaciens* Fad 82 spores grown on NAYE released more DPA during both HPP (beyond 8 min of holding) and PATP when compared to spores grown on TSAYE. The control (or untreated) samples had the almost the same amount of DPA in the supernatant. It is well known that the growth conditions and media affect the spore’s biochemical composition and its resistance (Christina et al., 2003). In order to determine the biochemical differences between spores grown on NAYE or TSAYE and treated by HPP or PATP, the spectra were analyzed by soft independent modeling of class analogy (SIMCA). It is a classification procedure based on principal component analysis and groups samples based on similarities in their biochemical composition. The top three principal components (PC) that explain the maximum amount of variance between the samples as three orthogonal axes are projected onto three dimensional space. Samples with large residual variance between them appear as separate clusters, are biochemically distinct and have an interclass distance (ICD; or Mahalanobis distance) of 3.0 or more between them (Kvalheim and Karstand, 1992).

The SIMCA class projection plot of *B. amyloliquefaciens* grown on NAYE and TSAYE and treated by HPP (35°C, 600 MPa, 70 min) and PATP (105°C, 600 MPa, 8
min) and the corresponding ICD values are shown in Figure 3.4. and Table 3.4., respectively. All the clusters in the SIMCA plot were visually distinct, implying a difference in biochemical composition between them. The IR bands that are different and the relative extent of difference are provided by the discriminating power plot. In essence, this plot shows which wavenumbers and the associated chemical groups are different between the samples being discriminated. Figure 3.5. highlights the IR bands that were different between B. amyloliquefaciens spores grown on the two media and treated by HPP or PATP. Interestingly, all the bands associated with DPA, 1412, 1378, 1440, 1616, 1570, and 1280 cm\(^{-1}\) (in the decreasing of discriminating power or relative importance), were found to be the most different among composition of all the samples. This data indicates a difference in the amount of DPA among the samples. All the DPA bands, except the band at 1412 cm\(^{-1}\), showed similar discriminating power upon processing regardless of the growth media. The band at 1412 cm\(^{-1}\) showed a higher discriminating power for spores grown on NAYE indicating that these spores have more difference in DPA composition during processing.

Spores grown on TSAYE were significantly different (ICD = 8.5) in composition, specifically DPA content, from those grown on NAYE. This observation suggests that the difference in the amount of DPA released by B. amyloliquefaciens grown on NAYE and TSAYE shown in Table 3.1. (NAYE > TSAYE) for the same treatment could possibly due to the difference in the initial DPA composition between spores grown on the two media. However, whether spores grown on TSAYE contain more but release lesser DPA than those grown on NAYE or spores grown on which media possesses higher initial
DPA content is not known. Recently, Ratphitagsanti and coworkers (Ratphitagsanti et al., 2009b) noted that *amyloliquefaciens* Fad 82 spores grown on NAYE were more resistant to PATP than those grown on TSAYE (\(D_{105°C \text{ and } 600 \text{ MPa}}\) were 1.4 ± 0.2 min and 1.0 ± 0.1 min, respectively). Furthermore, it is known that the concentration of spore DPA is directly proportional to its wet heat resistance. Hence, based on the above information, it can be speculated that the observed higher resistance and greater DPA release of *B. amyloliquefaciens* Fad 82 grown on NAYE compared to TSAYE, may be due to higher initial concentration of DPA in these spores.

### 3.4.4. Effect of processing

Marked differences were observed between the effect of HPP and PATP on the DPA release, irrespective of the growth media. As evident from Table 3.1., the amount of DPA released during PATP was significantly higher than during HPP treatment. Furthermore, PATP also showed a greater rate of release than HPP. For example, PATP (TSAYE - 12.59 ± 0.44 µM; NAYE - 49.41 ± 0.94 µM) achieved more DPA release during come-up-time (0.67 ± 0.08 min) than what was achieved by HPP after 70 min of holding (TSAYE - 9.44 ± 0.41 µM; NAYE - 38.82 ± 7.16 µM). Autoclaving (121°C, 0.1 MPa, 15 min) on the other hand released about 102.65 and 146.37 µM of DPA from *B. amyloliquefaciens* spores grown on TSAYE and NAYE, respectively. Earlier, Janssen and others (Janssen et al., 1958) reported that autoclaving (15 psi, 15 min) released essentially all DPA from the spores. However, our results indicate that the amount of DPA release is dependent on the sporulation media. Compared to PATP, autoclaving released almost the same amount of DPA from spores grown on TSAYE but less than
half from spores grown on NAYE. This further emphasizes more resistance of spores grown on NAYE and the combination of high pressure and high temperature is required to cause effective release of DPA from spores in a shorter time.

The difference in DPA release discussed above was also evident in the spectra of *B. amyloliquefaciens* Fad 82 spores before and after treatments, as shown in the SIMCA classification plot (Figure 3.4.). The treated samples, especially PATP-treated samples, were clustered away from the untreated samples. Sample classes T-C and T-H had an ICD of less than 3.0 (Table 3.4.), suggesting that no significant changes in DPA composition occurred in *B. amyloliquefaciens* spores grown on TSAYE after 70 min of HPP treatment at 35°C and 600 MPa. However, 8 min of PATP (105°C, 600 MPa) caused a significant change (ICD = 12.6) in DPA composition. On the other hand, spores grown on NAYE showed significant change in DPA composition during both HPP (ICD = 5.4) and PATP (ICD = 31.8). This observed difference is due to the availability of more DPA in spores grown on NAYE and greater lethality of PATP compared to HPP.

### 3.5. CONCLUSION

A rapid method based on FTIR spectroscopy was developed for the quantification of DPA release from bacterial spores during HPP and PATP. As evident from this research, FTIR provides biochemical information along with DPA release values, which extend the possibilities of its applications. NAYE produced higher DPA-containing and more resistant *B. amyloliquefaciens* spores than TSAYE and PATP released DPA from spores more effectively than HPP. FTIR spectroscopy could predict
the DPA release regardless of the growth media or high pressure processing conditions. However, few questions still remain unanswered and provides opportunities for further research. It is not known why the spores grown on NAYE were found to contain more DPA (and were more resistant) when compared to those grown on TSAYE. Furthermore, the exact difference in the mechanism of HPP and PATP is still unknown. FTIR spectroscopy combined with hydrophobic grid membranes was simple, convenient and rapid and offers a new research tool that could greatly simplify research on mechanism of bacterial spore inactivation.
3.6. REFERENCES


3.7. ACKNOWLEDGEMENT

The authors are thankful to the Center for Advanced Processing and Packaging Studies (CAPPS) and U.S. Army Natick Soldier Research, Development & Engineering Center for funding this project.
### 3.8. TABLES

#### Table 3.1. Amount of DPA released (µM ± standard deviation) by *Bacillus amyloliquefaciens* Fad 82 spores grown on TSAYE and NAYE during HPP (600 MPa and 35°C) and PATP (600 MPa and 105°C) treatment.

<table>
<thead>
<tr>
<th>Time</th>
<th>HPP</th>
<th>PATP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSAYE</td>
<td>NAYE</td>
</tr>
<tr>
<td>Control</td>
<td>4.95 ± 0.24</td>
<td>3.99 ± 0.92</td>
</tr>
<tr>
<td>0 min</td>
<td>5.36 ± 0.34</td>
<td>3.34 ± 0.45</td>
</tr>
<tr>
<td>5 min</td>
<td>6.75 ± 0.23</td>
<td>4.59 ± 1.03</td>
</tr>
<tr>
<td>8 min</td>
<td>6.79 ± 0.31</td>
<td>4.26 ± 0.90</td>
</tr>
<tr>
<td>30 min</td>
<td>8.04 ± 0.46</td>
<td>12.00 ± 0.15</td>
</tr>
<tr>
<td>70 min</td>
<td>9.44 ± 0.41</td>
<td>38.82 ± 7.16</td>
</tr>
<tr>
<td></td>
<td>3 min</td>
<td>99.47 ± 2.35</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>116.75 ± 3.01</td>
</tr>
<tr>
<td></td>
<td>8 min</td>
<td>132.92 ± 3.16</td>
</tr>
</tbody>
</table>

Table 3.2. DPA-related absorption bands in the FTIR spectra of PATP-treated *Bacillus amyloliquefaciens* Fad 82 spores.

<table>
<thead>
<tr>
<th>Wavenumbers (cm⁻¹)</th>
<th>Band Assignment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>~1280</td>
<td>amide III bands of proteins / DPA band</td>
</tr>
<tr>
<td>~1378</td>
<td>stretching bands of COO- group of Ca-DPA chelate</td>
</tr>
<tr>
<td>~1412</td>
<td>C-O-H in-plane bending of proteins</td>
</tr>
<tr>
<td>~1440</td>
<td>DPA pyridine ring vibration</td>
</tr>
<tr>
<td>~1570</td>
<td>C-N vibrations of the DPA ring</td>
</tr>
<tr>
<td>~1616</td>
<td>stretching bands of COO- group of Ca-DPA chelate</td>
</tr>
</tbody>
</table>

*Summarized from Goodacre et al., 2000; Perkins et al., 2005; Subramanian et al., 2006; Subramanian et al., 2007.*
Table 3.3. FTIR-predicted DPA concentrations (µM) released from *Bacillus amyloliquefaciens* Fad 82 spores grown in TSAYE treated by PATP (600 MPa, 105°C) with pulsing.

<table>
<thead>
<tr>
<th>PATP-Pulsing Treatment</th>
<th>Released DPA (µM)</th>
<th>Measured by Fluorometry</th>
<th>FTIR predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.00</td>
<td>5.60</td>
<td></td>
</tr>
<tr>
<td>1st Pulse holding (1.5 min)</td>
<td>108.82</td>
<td>96.64</td>
<td></td>
</tr>
<tr>
<td>1st pulse holding (1.5 min) + 1 min pause.</td>
<td>112.97</td>
<td>97.01</td>
<td></td>
</tr>
<tr>
<td>1st pulse holding (1.5 min) + 1 min pause + 2nd pulse come-up-time (1 min) + 2nd pulse holding (1.5 min)</td>
<td>117.71</td>
<td>101.81</td>
<td></td>
</tr>
<tr>
<td>Single pulse (3 min)</td>
<td>123.48</td>
<td>101.78</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4. Mahalanobis interclass distance values between treated and untreated *B. amyloliquefaciens* Fad 82 clusters in the SIMCA class projection plot.

<table>
<thead>
<tr>
<th>Clusters*</th>
<th>Interclass Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-C and N-C</td>
<td>8.5</td>
</tr>
<tr>
<td>T-C and T-H</td>
<td>2.0</td>
</tr>
<tr>
<td>T-C and T-P</td>
<td>12.6</td>
</tr>
<tr>
<td>N-C and N-H</td>
<td>5.4</td>
</tr>
<tr>
<td>N-C and N-P</td>
<td>31.8</td>
</tr>
</tbody>
</table>

*N – Spores grown on NAYE, T – Spores grown on TSAYE, H – HPP treatment (35°C, 600 MPa, 70 min), P – PATP treatment (105°C, 600 MPa, 8 min), and C – Control (untreated).
Figure 3.1. Fourier transform mid-infrared spectra of *Bacillus amyloliquefaciens* Fad 82 spore pellet, prepared on hydrophobic grid membrane and scanned in a FTIR microspectrometer. The insert shows the schematic of a typical hydrophobic grid membrane with applied bacterial spore samples.
Figure 3.2. Cross-validated (leave-one-out) partial least squares regression plot showing the correlation between fluorescence method and FTIR spectra for prediction of DPA concentration in treated *B. amyloliquefaciens* Fad 82 spore suspensions. A total of 4 PLSR factors were used to explain 97% of the model variance.
Figure 3.3. Partial least squares regression factor loadings plot for the first latent variable of the training set. Important wavenumbers that loaded significantly (both high and low) are marked. A greater the loading the greater is the importance of the wavenumber in the PLSR model.
Figure 3.4. Soft independent modeling of class analogy classification plot for *B. amyloliquefaciens* Fad 82 spores on NAYE and TSAYE, showing the biochemical difference between untreated and treated samples. N – Spores grown on NAYE, T – Spores grown on TSAYE, H – HPP treatment (35°C, 600 MPa, 70 min), P – PATP treatment (105°C, 600 MPa, 8 min), and C – Control (untreated).
Figure 3.5. Discriminating power plot highlighting the differences in FTIR absorbance bands between control (untreated) and treated (HPP – 35°C, 600 MPa, 70 min; PATP – 105°C, 600 MPa, 8 min) *B. amyloliquefaciens* Fad 82 spores grown on NAYE and TSAYE. A high discriminating power indicates a large difference in functional groups associated with the IR band.
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