CHAPTER-2.2

Spectroscopic analysis of *Cronobacter sakazakii* planktonic, biofilm and agar-surface associated cells
2.2.1 INTRODUCTION

Food borne illnesses and other bacterial related diseases are increasing dramatically. For this a rapid and precise detection of microorganisms is important. The traditional methods to detect microorganisms include morphological and biochemical tests. Apart from traditional methods genetic methods such as 16S ribosomal deoxyribonucleic acids “(DNA) or 16S ribosomal ribonucleic acid (RNA) gene has been used for bacterial identification” (Lu et al., 2011). However, such methods are time consuming and require expensive reagents and consumables. “Bioanalytical spectroscopy to study bacteria is an innovative technique that overcomes these drawbacks” (Lu et al., 2011).

“Fourier transform infrared (FTIR) and Raman spectroscopy provide fast and accurate detection of microorganisms. Both the techniques can provide “whole organism fingerprint” (Timmins et al., 1998). “The above two techniques are promising methods in food analysis and provide a broad range of biochemical properties about bacteria in a single spectrum” (Lu et al., 2011).

“FTIR spectroscopy can characterize bacteria as biochemical information about cellular components including proteins and peptides, carbohydrate, nucleic acids, phosholipids and murein are detectable” (Lu et al., 2011; Al-Qadiri et al., 2006). “Both infrared and Raman spectroscopy have been used in research” (Thygesen et al., 2003) for “microorganism detection and segregation” (Jarvis et al., 2008; Huang et al., 2010; Davis et al., 2010).

“Biofilms are the communities that are irreversibly attached to a surface, or to each other, and are embedded in a surrounding substance of extracellular polymeric substances (EPS) and exhibit different phenotypic characteristics with respect to their planktonic counterparts” (Donlan and Costerton, 2002). “Biofilm formation increases exopolysaccharide production and which protects the bacteria against a variety of antimicrobial agents and host attack” (Brown and Barker 1999). “FTIR and Raman Spectroscopy have been used to determine macromolecular composition of microbial biofilm matrices and also for monitoring the maturation and development of biofilm of bacteria, fungi, algae and protozoa” (Nivens et al., 1995). “A combination of Raman spectroscopy, electron microscopy and staining assay can reveal detailed information
of biofilms” (Du et al., 2012). Understanding the biofilm cell physiology will help in developing the strategies for their control.

“FTIR together with chemometrics has been used to study macromolecular compositions of pathogenic microorganism biofilms and also for monitoring the kinetics of maturation and development of biofilms from bacteria” (Nivens et al., 1995). Until now, “little work has been achieved on the application of spectral analysis of food borne pathogens colonization within biofilms” (Serra et al., 2008). “However, it is estimated that the method of bacterial growth within biofilms and attachment to abiotic surfaces may be similar between Gram-negative pathogens, for example, between Enterobacter sp., Pseudomonas sp., Escherichia sp, or Bordetella sp.” (Hall-Stoodly and Stoodly, 2002). Serra et al. (2008) “combined the proteomic approaches (MALDI-TOF mass spectrometry) and FTIR spectroscopy and studied that the putative acidic-type polysaccharide polymer was the most distinctive trait of B. pertussis life inside the biofilms and also how biofilms helps in B. pertussis pathogenesis”.

However, in general very little information is available on C. sakazakii biofilm cells compared to other biofilm forming pathogens such as Bordetella pertussis, Pseudomonas aeruginosa, and Escherichia coli O 157: H7, Salmonella spp. and Staphylococcus. “FTIR and Raman Spectroscopy can present a valuable tool in order to get an overview of planktonic and biofilm cell physiology” (Lu et al., 2011).

“Raman Spectroscopy provides advantages over FTIR spectroscopy because in the former sample need not be dried. Therefore Raman Spectroscopy has been extensively used in the biological sample analysis” (Beier and Berger, 2009). “Raman spectroscopy facilitates the analysis of hydrated biofilm cell samples” (Hudson and Chumanov 2009; Ileva et al., 2009) “as well as microorganisms embedded within it” (Andrews et al., 2010).

By employing the technique of FTIR and Raman spectroscopy we analysed the chemical differences in C. sakazakii grown as planktonic, AS and biofilm cells.
2.2.2 MATERIAL AND METHODS

1.2.2.1 Chemicals and reagents

“Chemicals and reagents used in the study were procured from Sigma Aldrich (USA), Bangalore Genei (India), Hi-Media (India), Merck (India), Qualigens (India), Galaxo (India) and Axygen (India)(Sharma and Prakash,2014)”.

2.2.2.2 Reviving of the culture

Cultures used in this study were revived in EE broth as described in section 1.2.4b.

2.2.2.3 Harvesting of planktonic, agar surface associated (AS) and biofilm cells

Planktonic, AS and biofilm cells were harvested as described in section 2.1.2.4, 2.1.2.5 and 2.1.2.6.

2.2.2.4 FTIR spectroscopic measurements

“FTIR bacterial spectra were obtained using a Varian 660-IR spectrophotometer, outfitted with DTGS (deuterated triglycine sulphate) detector and KBr beam splitter” (Bosch et al., 2006). The planktonic and biofilm cells suspension “spectra were recorded using HATR crystal” (Bosch et al., 2006). “Two hundred fifty-six scans were collected for good signal to noise ratio in the spectral range of 2000-650 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\)” (Bosch et al., 2006). The spectra of the planktonic and biofilm cells of the three samples (MTCC-2958, two C.sakazakii isolates) were recorded. Spectra of the planktonic cells of above three samples (Jal 1, Jal 5 and MTCC-2958) were recorded and averaged. “FTIR spectra were mean centred and baseline corrected” (Bosch et al., 2006). “A spectrum of saline solution (0.85% NaCl) was recorded and subsequently subtracted from the spectra of planktonic and biofilm cells suspension to carry out water subtraction” (Alex and Dupuis, 1989). “Water subtraction was attained by producing a flat baseline around 2200 cm\(^{-1}\), where water compensation mode is located” (Alex and Dupuis, 1989).

2.2.2.5 Raman instrumentation

“The biochemical characterization of C. sakazakii was examined using Raman spectroscopy (WITec, Ulm, Germany) equipped with UHTS-300 spectrophotometer”
(Du et al., 2012). “A wavelength of 532nm with a laser power of 2mW was focused onto the samples. The laser beam was focused on C. sakazakii planktonic and biofilm cells through a 20X objective. Raman scattering spectra were detected by a charge coupled-device (CCD) array detector. The size of each pixel was 16×16µm. WITec Control v1.5 software was used for instrumentation control and data collection (WITec, Ulm, Germany)” (Du et al., 2012). “A Raman spectrum was performed with a spectral range of 4000-500 cm$^{-1}$ for all the samples. For measurement at a single location, each full spectral measurement was for a 3-second integration time with 15 spectral accumulations (total integration time 45 seconds)” (Du et al., 2012).

2.2.3 RESULTS

“Figure 2.8 and 2.9 represent the FTIR and Raman spectra of C. sakazakii planktonic and biofilm cells respectively. Table 2.7 and 2.8 gives a summary of the main FTIR and Raman bands assigned to functional group of specific biological molecules respectively” (Sharma and Prakash, 2014).

2.2.3.1 FTIR spectroscopic analysis

“Comparative analysis of spectral data (Figure 2.8, Table 2.7) showed a significant increase in the intensity of absorption bands assigned to carbohydrate functional groups (spectral region: 993-1108 cm$^{-1}$) in AS and biofilm cells. In addition, bands assigned to the vibrational modes of carboxylate (spectral band: 1426 and 1341 cm$^{-1}$) also revealed such differences” (Sharma and Prakash, 2014).

“The difference involved the increase in the relative intensity of the band assigned to carboxylate group (1426 cm$^{-1}$), vibrational mode of ester (1745 cm$^{-1}$) in biofilm cells. There is higher intensity in the absorption bands of 1371 cm$^{-1}$, 1400 cm$^{-1}$ (Figure 2.8) provides an evidence for an increase in the production of uronic acids-containing polysaccharide by AS and biofilm cell” (Sharma and Prakash, 2014). According to Figure 2.8, “both amide I (1650 cm$^{-1}$) and amide II (1540 cm$^{-1}$) bands are present in planktonic, AS and biofilm cells spectra. However, there is an increase in intensity of a band (amide II) in AS and biofilm cells compared to planktonic cells” (Sharma and Prakash, 2014).
“AS cells produced spectra showing an increase in relative absorbance at 1,051 cm$^{-1}$ (C-OH stretching of alginate) and 1,259 cm$^{-1}$ (C-O stretching of the O-acetyl group in alginate); indicate the presence of O-acetyl groups in *C. sakazakii* AS cells” (Sharma and Prakash, 2014).

“Other differences involved the increase in the relative band intensity of fatty acids (1745 cm$^{-1}$) of biofilm cells” (Sharma and Prakash, 2014), Table 2.7; Figure 2.8).

2.2.3.2 Raman spectroscopic analysis

“Figure 2.9 represent the Raman spectroscopic analysis of planktonic and biofilm cells of *C. sakazakii* respectively. The band at 637 cm$^{-1}$, 625 cm$^{-1}$, 1254 cm$^{-1}$, 1677 cm$^{-1}$ represent tyrosine, phenylalanine, amide III and amide I respectively (Table 2.8). The typical characteristic peak at 1127 cm$^{-1}$ is a marker of carbohydrates C-C (skeletal), C-O, def (C-O-H). The distinct band at 1157 cm$^{-1}$ is assigned to the C-C structure of sarcinaxanthin and carotenoids. The band at 837 cm$^{-1}$ is characteristic of DNA, another band of 1459 cm$^{-1}$ is a marker band of lipids. The band at 1575-1578 cm$^{-1}$ depicts the guanine, adenine (ring structure). The spectral peak at 1006 cm$^{-1}$ depicts phenylalanine, substituted benzene derivative. The spectral peak at 752 cm$^{-1}$ depicts T ring structure. Another peak at 2935 cm$^{-1}$ denotes CH$_3$ and CH$_2$ structure and band at 3060 cm$^{-1}$ denotes C=C-H aromatic structure” (Sharma and Prakash, 2014).
**Table 2.7** Assignment of the main bands of the FTIR spectra

<table>
<thead>
<tr>
<th>Frequency (cm⁻¹)</th>
<th>Assignment</th>
<th>References</th>
</tr>
</thead>
</table>
| 720             | “C-H rocking of >CH₂ methylene”  
| 1200-900        | “C-OH str mode and C-O-C, C-O ring vibrations of carbohydrate (oligo, polysaccharide and alginate), C-O-P, P-O-P in polysaccharide of cell wall. P=O str (sym) of PO₂⁻ in nucleic acids”  
| 1375            | COO⁻ sym str | Nivens *et al.* (2001) |
| 1400-10         | “>C=O str (sym) of COO-and C-O bend from COO⁻”  
| 1540            | “Amide II ,N-H, C-N str of proteins and peptides”  
| 1650            | “Amide I , >C=O str and C-N bending of protein and peptide amide”  
| 1730-45         | “>C=O str of alkyl esters, fatty acids”  
### Table 2.8 Assignment of the main bands of the Raman spectra

<table>
<thead>
<tr>
<th>Frequency (cm⁻¹)</th>
<th>Assignment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>620</td>
<td>Phenylalanine</td>
<td>Maquelin et al. (2000)</td>
</tr>
<tr>
<td>640</td>
<td>Tyrosine</td>
<td>Maquelin et al. (2000)</td>
</tr>
<tr>
<td>752</td>
<td>T ring structure</td>
<td>Uzunbajakava et al. (2003)</td>
</tr>
<tr>
<td>~830</td>
<td>DNA</td>
<td>Maquelin et al. (2000)</td>
</tr>
<tr>
<td>~1004</td>
<td>Phenylalanine, substituted benzene</td>
<td>Maquelin et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>derivatives</td>
<td></td>
</tr>
<tr>
<td>1030-1130</td>
<td>Carbohydrate, mainly –C-C-(skeletal),</td>
<td>Schuster et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>C-O, def (C-O-H)</td>
<td></td>
</tr>
<tr>
<td>1155-1157</td>
<td>C-C str, of sarcinaxanthin, carotenoids</td>
<td>Rosch et al. (2005)</td>
</tr>
<tr>
<td>1254</td>
<td>Amide III</td>
<td>Uzunbajakava et al. (2003)</td>
</tr>
<tr>
<td>~1320</td>
<td>Amide III</td>
<td>Schuster et al. (2000)</td>
</tr>
<tr>
<td>1459</td>
<td>Lipids</td>
<td>van Manen et al. (2005)</td>
</tr>
<tr>
<td>1575-1578</td>
<td>Guanine, adenine (ring structure)</td>
<td>Maquelin et al. (2002)</td>
</tr>
<tr>
<td>1650-1680</td>
<td>Amide I</td>
<td>Maquelin et al. (2002)</td>
</tr>
<tr>
<td>2935</td>
<td>CH₃ and CH₂ structure</td>
<td>Maquelin et al. (2002)</td>
</tr>
<tr>
<td>3059</td>
<td>(C=C-H) aromatic structure</td>
<td>Maquelin et al. (2002)</td>
</tr>
</tbody>
</table>
Figure 2.8 Normalized FTIR spectra of cells harvested from planktonic (green line), AS (black line) and biofilms (blue line) of *C. sakazakii*. 
Figure 2.9 Normalized Raman spectra of cells harvested from planktonic (blue line) and biofilms (black line) of *C. sakazakii*.
2.2.3 DISCUSSION

FTIR spectroscopy

This work investigated the differences of the physiological responses of planktonic, agar-surface associated and biofilm cells of *C. sakazakii* using the techniques of FTIR and Raman spectroscopy. Qualitative FTIR analysis showed an increase in spectral bands assigned to carbohydrate functional groups proving that there is an increase in carbohydrate metabolism in AS and biofilm cells. “An increase in the level of exopolysaccharide production found in the biofilm cells in comparison to planktonic cells growing at a similar rate has been reported by” Vandevivere and Kirchman (1993). “Production of polysaccharide imparts survival strategies to bacterial biofilm and helps the bacteria to tolerate host defense mechanisms” (Serra et al., 2008). “The extracellular polymeric substance (EPS) accounts for over 90% of the biofilm content” (Flemming and Wingeender, 2010). “The components of EPS include proteins, carbohydrate, DNA and membrane vesicles” (Flemming et al., 2007). “Exopolysaccharide production is an energy consuming (anabolic) process, which can be down regulated by planktonic cells, when the exopolysaccharide are not required by the cells” (Costerton, 1999). “FTIR and Raman spectroscopy have been extensively used in microbiological analysis” (Beier and Berger, 2009). “The advantage of using these techniques is that they possess the ability to characterize chemical functional groups of biofilm architecture, which can be observed non-destructively” (Bosch et al., 2006; “Comparisons between the planktonic and biofilm cells tend to show a shift in functional groups relating to carbohydrates and proteins through FTIR spectra” (Bosch et al., 2006; Mukherjee et al., 2011).

“There is an increase in bands assigned to 1400 cm⁻¹ and 1371 cm⁻¹ in AS and biofilm cells spectra” (Sharma and Prakash, 2014) (Figure 2.8 and Table 2.7). “These bands at the specific frequencies have been assigned to uronic acid containing polysaccharide” (Bosch et al., 2006). “In *C. sakazakii* as in other species such as *Staphylococcus epidermidis*” (Shianu and Wu, 1998), “E.coli” (Yasud et al., 1994) and “Salmonella dysenteriae” (Qadri et al., 1994), “the biofilm matrix helps in pathogenesis and host defense mechanisms” (Brown and William, 1985). Similarly, an
“increase in the production of uronic acid in EPS contributes virulence characteristics of the biofilm cells” (Bosch et al., 2006). Fett et al., (1995) “reported that these acid sugars may help the stabilization of glycosidic linkages with the help of the carboxylic acid moiety, giving biofilm cells a higher resistance to acid hydrolysis”. “As a result, the chemical properties of EPS will increase the resistance of C. sakazakii AS and biofilm cells to acidic environment, as is seen in phagosomes during host attack, thus able to evade the host defense mechanism” (Pace et al., 1997). “The uronic acid containing polysaccharide produced by biofilm cells will help to colonize the bacteria in the enteric tracts and they may help them to aggregate and adhere to the cells” (Pace et al., 1997).

“In our experiment, AS and biofilm cells spectra produced an increase in the relative absorbance at 1,051 cm\(^{-1}\) (C-OH stretching of alginate) and 1,259 cm\(^{-1}\) (C-O stretching of the O-acetyl group in alginate)” (Sharma and Prakash, 2014). “Alginate production plays a role in the formation of microcolonies in-vitro and indicates that alginate may have a similar role in-vivo” (Nivens et al., 2001). “Alginate is a component of the biofilm cells matrix and this polymer may provide antiphagocytic effect” (Nivens et al., 2001).

“We observed a greater dominance of a band of 1745 cm\(^{-1}\) in biofilm cells in comparison to AS and planktonic cells” (Sharma and Prakash, 2014). Similarly Bosch et al., (2006) “studied that sessile cell spectra show 1.5-fold higher value for the band area of the band assigned to fatty acids as compared to planktonic cells”. Quiles et al., (2010) “observed a higher intensity of CH\(_2\) stretching band in a biofilms, with an increase in production of nucleic acids, in comparison to planktonic cells”.

**Raman spectroscopy**

“EPS is the biopolymer which consists of polysaccharides, proteins, nucleic acids and lipids and plays an important role during biofilm formation and maturation” (Flemming et al., 2010). Thus the spectral peak at 1127cm\(^{-1}\), increased in biofilm cells when compared to planktonic cells, and were assigned to carbohydrates” (Schuster et al., 2000). “The peaks were applied as marker to monitor the polysaccharide production during biofilm formation” (Schuster et al., 2000). Similar “results were obtained by” Kivens et al., (2006), “who found that a polysaccharide in biofilm cells
associated EPS of *Pseudomonas fluorescens* B52 was much higher (two to four fold) in comparison to planktonic cells”.

“Proteins, the major component of EPS matrix, provide a distinctive function for biofilm development” (Flemming *et al*., 2010). Hence, “in the present study peaks which were assigned to tyrosine (637 cm\(^{-1}\)), phenylalanine (625 and 1006 cm\(^{-1}\)), amide III (1254 cm\(^{-1}\), 1320 cm\(^{-1}\)), amide I (1677 cm\(^{-1}\)), increased in biofilm cells compared to planktonic counterpart” (Sharma and Prakash, 2014). Six peaks were used as marker to monitor the presence of proteins” (Sharma and Prakash, 2014).

“Polysaccharides present in cell-surface EPS help bacterial adhesion to glass surface” (Tsuneda *et al*., 2003). “In addition to polysaccharides, lipopolysaccharides” (Hall-Stoodly and Stoodly, 2002) and “proteins” (Gerlach and Hensel, 2007) present in “EPS may aid in initial attachment of bacteria to abiotic surfaces and thus help in biofilm formation” (Hall-Stoodly and Stoodly, 2002).

“The peak at ~837 cm\(^{-1}\) and 1483-1487 cm\(^{-1}\) in Raman Spectra is considered as a marker for nucleic acids” (Schuster *et al*., 2000). In the “present study the spectra at 837 cm\(^{-1}\) and 1483-1487 cm\(^{-1}\) increased in biofilm cells compared to planktonic cells indicating that DNA in biofilm cells was higher than that in the planktonic cells”. (Sharma and Prakash, 2014). This could be explained by the “release/ accumulation of extracellular DNA (e DNA) from the bacterial cells in the biofilm matrix” (Chao *et al*., 2011). “eDNA is a key structural component in the biofilm matrix and plays a variety of roles in biofilm development, adhesion” (Harmsen *et al*., 2010), “cohesion” (Jermy *et al*., 2010) and “exchange of genetic material” (Flemming *et al*., 2010). Similar studies were conducted by Andrew *et al*., (2010) “showing that the intensity of nucleic acids was much greater in biofilms in contrast to planktonic cells”.

In the “present study the biofilm cells also consist of lipids and could be identified according to the spectral peak at 1459 cm\(^{-1}\)” (Sharma and Prakash, 2014) , (Table 2.8 and Figure 2.9). This spectral peak had a “higher intensity in biofilm cells than in plankonic cells. *C. sakazakii* is a gram negative bacteria and lipopolysaccharide is the chief component of the outer membrane of Gram-negative bacteria” (Walker *et al*., 2004). “It was also observed that carotenoids were also the constituent of biofilm cells according to the spectral peak obtained at 1157 cm\(^{-1}\)” (Sharma and Prakash, 2014).
(Table 2.8 and Figure 2.9). Du et al., 2012 “conducted Raman imaging and showed an even distribution of carotenoids on the biofilm EPS”. “It is well known that for the survival of bacteria in harmful environmental conditions, carotenoids plan an important role in scavenging reactive oxygen species” (Johler et al., 2010).

“Our study showed that differences in the cellular composition, of planktonic and biofilm cells reflecting in spectral feature helped in the characterization of C. sakazakii biofilm cells” (Sharma and Prakash, 2014).