JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

COMBINED USE OF FOURIER TRANSFORM INFRARED AND RAMAN SPECTROSCOPY TO STUDY PLANKTONIC AND BIOFILM CELLS OF *CRONOBACTER SAKAZAKII*

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ARTICLE INFO

Received 8. 10. 2013
Revised 25. 11. 2013
Accepted 10. 12. 2013
Published 1. 2. 2014

Regular article

OPEN ACCESS

ABSTRACT

Cronobacter sakazakii is an opportunistic pathogen, which causes necrotizing enterocolitis, bacteriaemia and infant meningitis. It has the ability to form biofilm on food contact surfaces, creating food safety risks. In this work, the phenotypic expression of planktonic and biofilm was studied by Fourier transform infrared (FTIR) and Raman spectroscopy. FTIR spectra of the biofilm cells exhibited higher intensity in the absorption bands assigned to polysaccharides, amide I, amide II vibrational mode of ester and carboxylate group. Raman spectra of the biofilm cells showed higher intensity in the absorption band assigned to tyrosine, amide III, carbohydrates, carotenoids, DNA and lipids. Understanding the chemical properties of planktonic and biofilm cells employing the two techniques helped to decipher the differences in the chemical composition between planktonic and biofilm cells. This can promote a better understanding of the persistence, survival and resistance of the biofilm cells.

Keywords: *C.sakazakii,* biofilm cells, planktonic, spectroscopy, exopolysaccharide

INTRODUCTION

Food borne illnesses and other bacterial related diseases are increasing dramatically. For this a rapid and precise detection of microorganism is important. *C.sakazakii* is an opportunistic food borne pathogen and is associated with infections in neonates, infants and persons with congenital and acquired immunodeficiency. Traditional methods to detect microorganism includes 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. However, such methods are time consuming and require expensive reagents and the consumables. Bioanalytical spectroscopy to study bacteria is an innovative technique that overcomes these drawbacks.

Fourier transform infrared (FTIR) and Raman spectroscopy provides fast and accurate detection of microorganism. Both the techniques can provide "whole organism fingerprint" (**Timmins *et al.*, 1998**). The above two techniques are promising methods in food analysis and provides a broad range of biochemical properties about bacteria in a single spectrum. FTIR spectroscopy can characterize bacteria as biochemical information about cellular components including proteins and peptides, carbohydrate, nucleic acids, phosholipid and murein are detectable **(Lu *et al.*, 2011; *Lin et al.*, 2009; Al-Qadiri *et al.*, 2006)**. Both infrared and Raman spectroscopy has been used in research **(Thygesen *et al.*, 2003)**, microorganism detection and segregation (Jarvis and Goodacre 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 increase exopolysaccharide production and which protect the bacteria against a variety of antimicrobial agent 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 biofilm **(Du *et al.*, 2012)**. Understanding the biofilm cell physiology will help in developing the strategies for their control. However, in general very little information is available on *C.sakazakii* biofilm cells compared to other biofilm forming pathogens such as *Bordutella 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.

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; Choo-Smith *et al.*, 2001)**. Raman spectroscopy facilitates the analysis of hydrated biofilm cell samples **(Hudson and Chumanov 2009; Ivleva *et al.*, 2009)** as well as microorganisms embedded within it **(Andrews *et al.*, 2010)**.

The aim of the present work was to analyse, through FTIR and Raman spectroscopy, the chemical differences between planktonic and biofilm cells of *C.sakazakii*.

MATERIALS AND METHODS

Bacterial culture preparation

A total of 20 milk and cottage cheese was collected from different areas of Agra city. The sample included raw buffalo milk (n=15), cottage cheese (n=5). Cottage cheese samples were collected from local vendors of Agra. About 0.5 g or 0.5 ml of milk and cottage cheese was added to 4.5 ml of Enterobacter Enrichment (EE) broth and incubated overnight at 37° C. Culture streaked on Violet Red Bile Glucose Agar (VRBGA), and obtained colonies were confirmed as *C. sakazakii* using traditional microbiological methods (Gram's staining, Methyl red test, Voges-Proskauer test, nitrate test, indole production test, catalase test, oxidase test, citrate utilization test, triple sugar iron test, gas production, H_2S production test, DNase test, motility test and a sugar fermentation tests). Biochemically confirmed isolates were confirmed as *C.sakazakii* using primers ESSF and ESSR **(Nair and Venkitanarayanan, 2006)** to amplify unique *omp A* gene specific to *C.sakazakii*. The standard bacterial isolate (MTCC-2958) used in the study was procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India and two *C.sakazakii* isolates (biochemically and molecularly confirmed as *C.sakazakii*) were further analysed by FTIR and Raman Spectroscopy.

Table 1 Gram's staining and biochemical characterization of *C. sakazakii* (+ sign is for positive biochemical reaction , - sign is for negative biochemical reaction).

S.No.	Biochemical Test		Result
1	Gram's staining		-
2	Methyl Red		-
3	Voges-Proskauer		+
4	Nitrate		+
5	Indole Production		-
6	Catalase		+
7	Oxidase		-
8	Citrate Utilization		+
9	TSI		-
10	Gas Production		+
11	H_2S Production		-
12	Sugar Tests		
	(a)	Adonitol	-
	(b)	Arabinose	-
	(c)	Glucose	+
	(d)	Maltose	+
	(e)	Raffinose	+
13	DNase test		+
14	Motility test		+

Reviving of the culture

The two *C.sakazakii* isolates and *C.sakazakii* standard (MTCC-2958) were grown at 37° C for 24 hours in Enterobacter Enrichment (EE) broth and streaked on VRBGA. Typical purple coloured colonies appeared on VRBGA after 24 hours incubation at 37°C.

Detection of biofilm cell formation

Biofilm formation was determined as described by **Mathur *et al*. (2006)**. EE broth (3ml) was inoculated with loop full of microorganisms from overnight culture plates and incubated for 24 hours at 37° C. Culture glass tubes were decanted and washed with 0.85% sodium chloride solution to remove 'planktonic' bacteria and dried. After drying tubes were stained with 0.1% crystal violet. Excess stain was removed and tubes were washed with 0.85 % sodium chloride and dried in an inverted position and examined for biofilm formation. Visible film lining the wall and the bottom of the tube was considered as biofilm formation.

Harvesting of planktonic cells

C.sakazakii standard (MTCC-2958) and *C.sakazakii* isolates were inoculated in 3ml of EE broth with a loop full of confirmed *C.sakazakii* isolates from overnight culture plates and incubated for 24 hours at 37°C. The culture was centrifuged at room temperature for 15 minutes at 4000g to harvest bacterial cells. Media component and bacterial metabolites were removed by suspending the pellet in 0.85 % sodium chloride solution and centrifuged again; this step was repeated twice. The supernatant was discarded and the wet pellet was suspended in 300 µl of 0.85 % sodium chloride solution and mixed to obtain a homogenous cell distribution.

Harvesting of the biofilm cells

C.sakazakii standard (MTCC-2958) and *C.sakazakii* isolates were inoculated in 3ml of EE broth with a loop full of confirmed *C. sakazakii* from overnight culture plates and incubated for 24 hours at 37°C. After incubation the glass test tubes were decanted and washed twice with 0.85 % sodium chloride solution remove planktonic cells. The 0.85 % sodium chloride solution was added to the test tube and vortexed to dislodge the biofilm cells. Biofilm cells were centrifuged at room temperature for 15 minutes at 4000 g. The supernatant was discarded and the wet pellet was suspended in 300µl of 85 % sodium chloride solution and mixed to obtain a homogenous cell distribution.

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. The planktonic and biofilm cells suspension spectra were recorded using HATR crystal. 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}. 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 were recorded and averaged. FTIR spectra were mean centred and baseline corrected. 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 substraction (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).

Raman Instrumentation

The biochemical characterization of *C. sakazakii* was examined using Raman spectroscopy (WITec, Ulm, Germany) equipped with UHTS-300 spectrophotometer. 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 1600- by 200 pixel CCD (charge coupled-device) array detector. The size of each pixel was 16×16 µm. WITec Control v1.5 software was used for instrumentation control and data collection (WITec, Ulm, Germany). 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).

RESULTS

Out of the total of 20 milk and cottage cheese, 100 isolates were obtained, of which 2 were biochemically and molecularly confirmed as *C.sakazakii,* included one isolate from raw buffalo milk, one from cottage cheese. Table 1 depicts the results of microbiological tests used for confirming these two isolates. Primers ESSF and ESSR were effectively used to amplify *ompA* (469 bp) gene unique to *C.sakazakii*. Visible stained cells along the lining and bottom of the glass test tube confirmed the biofilm formation by MTCC-2958 and the two *C.sakazakii* isolates. Figure 1 and Figure 2 represent the FTIR and Raman spectra of *C.sakazakii* planktonic and biofilm cells respectively. Table 2 and 3 gives a summary of the main FTIR and Raman bands assigned to functional group of specific biological molecules respectively.

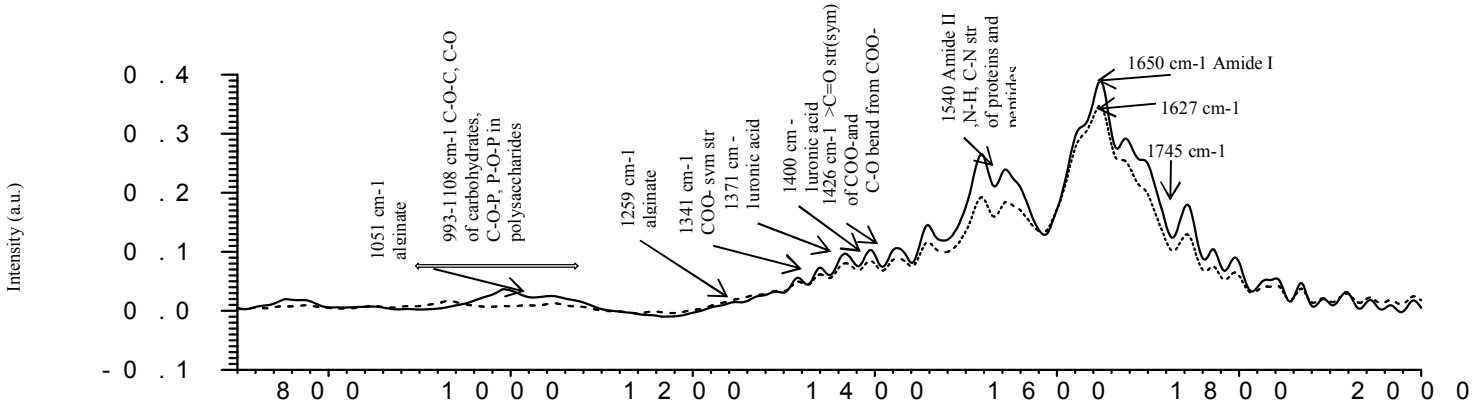

Figure 1 Normalized FTIR spectra of planktonic (dotted line) and biofilm (continuous line) cells of *C.sakazakii.*

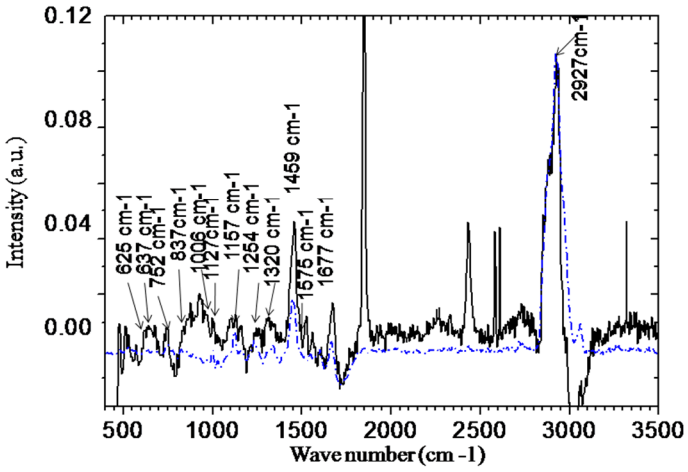

Figure 2 Normalized Raman spectra of planktonic (dotted line) and biofilm (continuous line) cells of *C.sakazakii.*

Table 2 Assignment of the main bands frequency in FTIR spectra

Frequency (cm^{-1})	Assignment	References
720	C-H rocking of $>CH_2$ methylene	**Naumann, (2000)**
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 PO_2^- in nucleic acids	**Naumann, (2000)**
1375	COO^- sym str	**Nivens *et al.* (2001)**
1400-10	>C=O str(sym) of COO-and C-O bend from COO-	**Naumann, (2000)**
1540	Amide II ,N-H, C-N str of proteins and peptides	**Naumann, (2000)**
1650	Amide I , >C=O str and C-N bending of protein and peptide amide	**Naumann, (2000)**
1730-45	>C=O str of alkyl esters, fatty acids	**Naumann, (2000)**

Table 3 Assignment of the main bands frequency in Raman spectra

Frequency (cm^{-1})	Assignment	References
620	Phenylalanine	**Maquelin *et al.* (2000)**
640	Tyrosine	**Maquelin *et al.* (2000)**
752	T ring structure	**Uzunbajakava et al. (2003)**
~830	DNA	**Maquelin *et al.* (2000)**
~1004	Phenylalanine, substituted benzene derivatives	**Maquelin *et al.* (2000)**
1030-1130	Carbohydrate, mainly –C-C-(skeletal), c-O, def (C-O-H)	**Schuster *et al.* (2000)**
1155-1157	C-C str, of sarcnaxathin, carotenoids	**Rosch *et al.* (2005)**
1254	Amide III	**Uzunbajakava *et al.* (2003)**
~1320	Amide III	**Schuster *et al.* (2000)**
1459	Lipids	**van Manen *et al.* (2005)**
1575-1578	Guanine, adenine (ring structure)	**Maquelin *et al.* (2002)**
1650-1680	Amide I	**Maquelin *et al.* (2002)**
2935	CH3 and CH2 structure	**Maquelin *et al.* (2002)**
3059	(C=C-H) aromatic structure	**Maquelin *et al.* (2002)**

FTIR Spectroscopic analysis

Comparative analysis of spectral data (Figure 1, Table 2) showed a significant increase in the intensity of absorption bands assigned to carbohydrate functional groups (spectral region : 1108-993 cm^{-1}) in biofilm cells. In addition, bands assigned to the vibrational modes of carboxylate (spectral band: 1426 and 1341 cm^{-1}) also revealed such differences.The difference involved the increase in the relative intensity of the bands of 1627 cm^{-1} (asymmetrical stretching of carboxylate ion), bands assigned to vibrational mode of ester (1745 cm^{-1}). There is higher intensity in the absorption bands of 1371 cm^{-1}, 1400 cm^{-1} (Figure 1) provides an evidence for an increase in the production of uronic acids-containing polysaccharide by biofilm cell (Bosch *et al.,* 2006). According to Figure 1, both amide I (1650 cm^{-1}) and amide II (1540 cm^{-1}) bands are present in biofilm and planktonic cells spectra, there is an increase in intensity of a band in biofilm cells compared to planktonic cells. Biofilm 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* of biofilm cells. These bands have been previously reported in FTIR spectra of *P.aeuroginosa* as a major constituent in biofilm cells architecture (Nivens *et al.,* 2001). Another difference involved the increase in the relative intensity of the band of fatty acids (1743 cm^{-1}, Table 2, Figure. 1) increased in biofilm cells.

Raman Spectroscopic analysis

Figure 1 and 2 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 3). The typical characteristic peak at 1127 cm^{-1} is a marker of carbohydrates C-C (skeletal), C-O, def (C-O-H) **(Schuster *et al.*, 2000).** The distinct band at 1157 cm^{-1} assigned to the C-C structure of sarcinaxanthin and carotenoids **(Rosch *et al.*, 2005)**. The band at 837 cm^{-1} was characteristic of DNA **(Deng et al., 1999)**; another band of 1459 cm^{-1} is a marker band of lipids **(van Manen *et al.*, 2005)**. The bands at 1575-1578 cm -1 depict the guanine, adenine (ring structure) **(Maquelin *et al.*, 2002).** The band at 1483-1487 cm^{-1} was derived from nucleic acids **(Schuster *et al.*, 2000).** The spectral peak at 1006 cm^{-1} depicts phenylalanine, substituted benzene derivative **(Maquelin *et al.*, 2002).** Another peak at 2935 cm^{-1} denote CH_3 and CH_2 structure **(Maquelin *et al.*, 2002)** and band at 3060 cm^{-1} denote C=C-H aromatic structure **(Maquelin *et al.*, 2002).**

DISCUSSION

FTIR spectroscopy

This work investigated the differences of the physiological responses of planktonic and biofilm cells of *C.sakazakii* using the techniques of Raman and FTIR 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 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 have been reported by **Vandevivere and Kirchman (1993)** and **Evans *et al.* (1994)**. Production of polysaccharide imparts survival strategies to bacterial biofilm and helps the bacteria to tolerate host defence 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).** Exoplysaccharide 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, 1985).** FTIR and Raman spectroscopy have been extensively used in microbiological analysis **(Choo-smith *et al.*, 2001; Wagner *et al.*, 2009; Beier and Berger 2009).** The advantage of using this technique is that they possess the ability to characterize chemical functional groups of biofilm architecture, which can be observed non-destructively. Comparisons between the planktonic and biofilm cells tend to show a shift in functional groups relating to carbohydrates and proteins have been shown through FTIR – spectra **(Schmitt and Fleming 1998; Bosch *et al.*, 2006; Mukherjee *et al.*, 2011).** There is an increase in bands assigned to 1400 cm^{-1} and 1371 cm^{-1} in biofilm cells spectra. These bands at the specific frequencies were assigned to uronic acid containing polysaccharide **(Bosch *et al.*, 2006)**. In *C. sakazakii* as in other species *Pasteurella multocida* (Chung *et al*., 2001), *Staphylococcus epidermidis* **(Shianu and Wu 1998)**, *E.coli* **(Yasud *et al.*, 1994)**, *Salmonella dysenteriae* **(Qadri *et al.*,1994)** and *Pseudomonas aeruginosa* **(Meluleni *et al.*,1995)**, the biofilm matrix helps in pathogenesis and host defense mechanisms **(Brown and William, 1986).** 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.* (1915)** 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* biofilm cells to acidic environments, as is seen in phagosomes during host attack, thus able to evade the host defense mechanism. 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.*, 2007)**.

In our experiment, the biofilm cells spectra produced an increase in the relative absorbance at 1060 cm^{-1} (C-OH stretching of alginate) and 1,250 cm^{-1} (C-O stretching of the O-acetyl group in alginate). Alginate production plays a role in the formation of microcolonies *in-vitro* and indicates that alginate may have a similar role *in-vivo*. 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 1743 cm^{-1} in biofilm cells in comparison to planktonic cells. Similarly **Bosch *et al.* (2006)** studied that sessile cell spectra showed 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 biofilm cells, 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. 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. Six peaks are used as marker to monitor the presence of proteins

Polysaccharides present in cell-surface EPS help bacterial adhesion to glass surface **(Tsuneda *et al.*, 2003)**. In addition to polysaccharides, lipopolysaccharides **(Doonlan 2002; 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.

The peak at ~837 cm^{-1} and 1483-1487 cm^{-1} in Raman Spectra is considered as a marker for nucleic acid **(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 in comparison to planktonic cells. This could be explained by the release/ accumulation of extracellular DNA (eDNA) from the bacterial cells in the biofilm matrix **(Chao *et al.*, 2011)**. The 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 biofilm cells in contrast to planktonic cells. Biofilm cells also consist of lipids and could be identified according to the spectral peak at 1459 cm^{-1}, (Table 3, Fig. 2). 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 is well known that for the survival of bacteria in harmful environmental condition, carotenoids play an important role and help in scavenging reactive oxygen species **(Johler *et al.*, 2010)**. In the present study the spectra at 1157 cm^{-1} (Table 3, Figure 2) increased in biofilm cells compared to planktonic cells indicating that carotenoids are the constituent of biofilm cells as previously reported by **Du *et al.* (2012); Ivelva *et al.* (2009) and Andrew *et al.* (2010)**. Our study showed that differences in the cellular composition of planktonic and biofilm cells, reflecting in spectral feature can help in the characterization of *C. sakazakii* biofilm cells.

CONCLUSIONS

FTIR and Raman Spectroscopy are relatively fast and simple techniques. These are non destructive and sensitive techniques that require very small amount of samples. Spectra obtained from the two techniques provide information about the whole bacterial cell composition. It is relatively less expensive for bacterial cell composition in comparison to other techniques. The study using these techniques demonstrated that *C.sakazakii* biofilm grown cells are chemically different from planktonic cells. Nevertheless, further studies are needed to characterize and elucidate the biofilm cells of *C. sakazakii* grown in actual food environment conditions.

Acknowledgement: The work was supported by the UGC Meritorious Student Fellowship and Dayalbagh Educational Institute, Dayalbagh, Agra. We also thank the Dr. Ranjana Mehrotra, National Physical Laboratory, New Delhi for Raman and FTIR spectroscopic instrumentation.

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