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Research Article

ANALYSIS OFBIOFILM AND FREE CELL COMPONENTS OF *Streptococcus agalactiae* ISOLATED FROM BOVINE MASTITIS USING FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

[1,*] Nasim Sohail, M., [2]Rathnamma, D. and [3]Avinash, B.

[1]Department of Animal Science, Faculty of Agriculture, Kandahar University, Kandahar, Afghanistan
[2]Department of Veterinary Microbiology, Veterinary College, Hebbal, Bangalore, India.
[3]Department of Veterinaryharmacology, Veterinary College, Hebbal, Bangalore, India

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ABSTRACT

Fourier Transform Infrared (FTIR) technique is a valuable tool for investigation of biochemical composition of bacterial biofilms.Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy was used to analyze and compare *Streptococcus agalactiae* bio film exopolysaccharide (EPS) matrix and free cell components. As per the standardization of growth kinetics and biofilm formation of*S.agalactiae*in our earlier studies, biofilm was grown in 0.625 per cent Luria-Bertani (LB) broth with one per cent glucose on 0.3 per cent bentonite clay as inert surface for 3 days and 2.5 per cent LB glucose on 0.5 mm glass beads as inert surface for 48 hrs. Free cells were grown in 2.5 per cent LB glucose for 24 hrs. Biofilm EPS and free cell componenets were subjected for ATR-FTIR spectroscopy. The results revealed marked differences in the chemical composition of the biofilm EPS and free cell components. Differences were observed particularly in the carbohydrate region between 1200-900 cm^{-1} wavenumber and the protein region. Interestingly, the *S. agalactiae* biofilms grown on bentonite clay and glass beads showed increasing IR signalling intensities at the polysaccharide spectral regions, instead, the free cells showed decreasing IR signalling intensities at this region, whereas free cells revealed prominent peaks of proteins. This finding clearly indicated that the polysaccharide concentrations were more in biofilms compared to free cell components. These temporal differences reflect the presence of excess amount of polysaccharides in biofilms and may be related to exopolysacharide production during biofilm development.This is the first report of *S. agalactiae* biofilm analysis using FTIR spectroscopy.

INTRODUCTION

Streptococcusspeciesare one of the most important bacteria associated withmastitis in bovines. *Streptococcus agalactiae*is an obligate parasite of the bovine mammary gland with herd prevalence rates ranging from 11 per cent (Schoonderwoerd *et al.,* 1993) to 47 per cent (Goldberg *et al.,* 1991). *In-vitro* studies have shown that *S. agalactiae* isolated from both animal and humans are potential biofilm producers (Merle *et al.,* 2002; Ghiorghi*et al.,* 2009 and Cira*et al.,* 2010). Earlier, these microorganisms were studied by culturing in highly enriched liquid or solid media. However, bacteria exist within natural systems are entirely differ from artificially grown laboratory strains. Sessile bacteria growing on surfaces have nutrient limitations and so growing more slowly whereas planktonic bacteria in culture media have unnatural access to nutrients, multiply rapidly and often are highly motile. Hence, planktonic bacteria are more susceptible to the effects of antibiotics and to environmental and host factors. Conversely, sessile bacteria are able to resist or evade such destructive factors by forming aggregates, altering their physiology and taking advantage of deficiencies in the host clearance mechanisms (Costerton *et al.,* 1995 and Mah *et al.,* 2001).Many persistent and recurrent bacterial infections have been attributed to the formation of 'biofilm' or polymeric matrices produced by bacterial colonies adhering to a biologic or abiotic surface. A biofilm matrix is composed of microbial cells, polysaccharides, water and other extra cellular products, all of which allow the biofilm matrix to be hostile to numerous microenvironments (Costerton *et al.,* 1999; Mah *et al.,* 2001 and Sutherland, 2001).Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy was used to analyze and compare *Streptococcus agalactiae* biofilm exopolysaccharide (EPS) matrix and free cell components.Fourier transform infrared (FT-IR)

Corresponding author: Nasim Sohail, M.,
Department of Animal Science, Faculty of Agriculture, Kandahar University, Kandahar, Afghanistan.

spectroscopy is one of the important technologies that have been gaining a wide application for the detection and analysis of microbial and organic compounds. Infrared spectroscopy measures the absorption, transmission, or reflection of mid-infrared (IR) radiation with wavelengths ranging from 2.5 to 25 μm resulting from the interaction of the electric dipole movement of the molecule by the IR radiation. Fourier transform infrared spectroscopy was used earlier by many researchers for the evaluation of the components of microbial biofilms such as *Pseudomonas* species (Michele, 2009), acidophilic microbial biofilms from mines *(*Yongqin *et al.,* 2010), marine bacterial biofilms(Muthusamy *et al.,* 2011) and bacterial biofilms of oral dental plaques of humans (Kirti, 2011 and Sheetal, 2011).However, there are no reports on the use of FT-IR spectroscopy to study the biochemical changes associated with biofilm formation of*S.agalactiae*. In the present study an attempt was made to analyse the biofilm and free cells components using FT-IR spectroscopy, the result of this study clearly indicated that the polysaccharide concentrations were more in biofilms compared to free cell components. These temporal differences reflect the presence of excess amount of polysaccharides in biofilms and may be related to exopolysacharide production during biofilm development. This is the first report of *S. agalactiae* biofilm analysis using FTIR spectroscopy.

MATERIALS AND METHODS

*Streptococcus agalactiae*isolates

Streptococcusagalactiae isolated from bovine mastitis cases and maintained at NAIP Subproject on Bovine mastitis, Department of Veterinary Microbiology, Veterinary College, Bengaluruwereutilized for the study.

Analysis of *S. agalactiae* biofilm EPS and free cell components using FTIR spectroscopy

Growing of *S. agalactiae*biofilm for EPS extraction.

In the previous study (Nasim,2012),the maximum biofilm formation by *S. agalactiae* was standardized, biofilmEPS were extracted from *S. agalactiae*grown in 0.625 per cent LB glucose with 0.3 per cent bentonite clay as an inert surface for 3 days and 2.5 per cent LB glucose with 0.5mm glass beads as an inert surfacefor two days.The freecell components were extracted from *S. agalactiae*grown in 2.5 per cent LB glucose for 24 hrs.Exopolysaccharide of biofilm and of free cells of *S. agalactiae* was extracted as per the procedure described by Yongqin*et al.* (2010).

FT-IR analysis of biofilm and free cell components

Fourier transforminfrared spectroscopy was carried out as per the procedure described by Yongqin*et al.* (2010).Biofilm EPS and free cell components of *S. agalactiae* were analysed using a Nicolet 6700 FTIR spectrometer (Thermo Fisher Scientific Inc., Madison, WI, USA) controlled by OMNIC^{TM}8.1.11 software with smart Orbit^{TM}, a horizontal single ATR accessory with type II A diamond crystal mounted in a tungsten carbide plate. The spectrometer was consisting of a DTGS KBr detector and KBr beam splitter. The sample was placed on the diamond crystal of Smart Orbit and uniformly pressed by turning pressure knob (45 lbs) of swivel pressure tower connected with powder tip accessory.A background scan was recorded prior to acquisition of first sample spectra. Further background scan were recorded for every 30 min time interval. All the samples and background spectra were acquired by scanning 32 times with the resolution of 2.0 cm^{-1} and data spacing of 0.964234 cm^{-1}. All spectra were acquired by setting the instrument for automatic atmosphere suppression and no other corrections were applied during spectral acquisition. The final format of the spectra was absorbed *v/s* wave number (cm^{-1}) with the spectra range from 400-4000 cm^{-1} (Mid IR). The acquired spectra were subjected to automatic base line correction, the corrected spectra were used for peak analysis. Each standard peak was properly labelled and minimum of three spectra for each standard peak was acquired for analysis.

RESULTS

Analysisof biofilm and free cell components *S. agalactiae*by (FT-IR) spectroscopy

The exopolysaccharides (EPS) matrix of biofilms and cellular components of free cells of *S. agalactiae* were extracted and subjected forAttenuated Total Reflectance Fourier TransformInfraredSpectroscopy (ATR- FT-IR).The infrared spectroscopic fingerprints of EPS of biofilms and free cell components showed both major and subtle chemical compositional changes between the biofilm's EPS and free cell components.

FT-IR analysisofEPS of *S. agalactiae*biofilm grown on bentonite clay

TheFT-IR spectral peaks identified and characterized in biofilm EPS grown on bentonite clay were at wavenumber3274cm^{-1} (amide A, N-H stretching in protein), 1651cm^{-1} (amide -I in protein), 1535cm^{-1}(amide II inprotein), 1456cm^{-1} (νsym C=O of COO^-δ as CH_3 of methyl groups of protein), 1107cm^{-1}(νC-O, νC-C ring in polysaccharides), 1022cm^{-1}(δC-O, δ C-C, δ O-CH, ring in polysaccharide), 991cm^{-1} (νC-O, νC-C Vibration, polysaccharides)and 906cm^{-1}(Phosphodiester stretching region-polysaccharides).The major peaks identified and characterized as polysaccharides in EPS of biofilms grown on bentonite clay were at wavenumber1107cm^{-1}, 1022cm^{-1}, 991$cm^{-1}$and 906cm^{-1}. Among these, 1022cm^{-1}, 991cm^{-1} and906cm^{-1} polysaccharidepeaks were prominent, whereas the polysaccharide peak at wavenumber1107cm^{-1} was weak.The peaks specific for proteins identified were at wavenumbers3274 cm^{-1} , 1651 cm^{-1}, 1535 cm^{-1} and 1456 cm^{-1} in biofilm EPS grown on bentonite clay and were not prominent (Fig. 1 and Table 1).

FT-IR analysis of EPS of *S. agalactiae*biofilm grown on glass beads

The peaks identified and characterized in biofilm EPS grown on glass beads were at wavenumber3272 cm^{-1} (amide A, N-H stretching,protein), 1744 (ν C=O, Ester- polysaccharide) 1652cm^{-1} (Amide I – αHelix in protein), 1535cm^{-1}(amide IIprotein) 1456cm^{-1} (νsym C=O of COO^-δ as CH_3 of methyl groups of protein), 1022 cm^{-1}(δ C-O, δC-C, δ O-CH, ring-polysaccharide), 994cm^{-1} and 907cm^{-1} (Phosphodiesterstretching regionpolysaccharide).The major

peaks identified and characterized in biofilm EPS grown on glass beads were 1022 cm^{-1}, 994 cm^{-1} and 907 cm^{-1} which were mainly polysaccharides and all of them were very predominant in biofilm grown under this particular condition. The weak peaks identified were at wavenumber3272 cm^{-1} ,1652 cm^{-1}, 1535 $cm^{-1}$and 1456 cm^{-1}, which are specific for proteins were not prominent in biofilm EPS grown on glass beads (Fig.2 and Table 1).

1456cm^{-1} (νsym C=O of COO^{-}δ as CH_3 of methyl groups of protein), 1412 cm^{-1} (νC-N, δ N-H, δ C-H – Protein), 1215cm^{-1}(amide III in protein), 1161 cm^{-1}(ν C-O / C-OH in polysaccharide),1038 cm^{-1}(ν C-C, (ν CH_2OH), ν C-O+ δ C-O,ν S-O, Cysteine monoxide), 972cm^{-1} shoulder (ν as N-CH_3,O-CH_3inpolysaccharide) and 719cm^{-1} (C-Hvibration > CH_2, N–H Amide IV in protein). The major peaks specific for proteinswereat wavenumber3273 cm^{-1} , 3084 cm^{-1}, 1624 cm^{-1}

Table 1. FTIR spectral peaks of S. agalactiae biofilm EPS on BC and glass beads and free cells components

S/N	BF on BC	BF on GB	Free cell components	Spectral peaks assignment (Stuart, 1997; Movasaghi*et al.*,2008 andNaumann*et al.*, 2009)	Component
1	3274	3272	3273	Amide A (N-H stretching)-	Protein
2	-	-	3084	Amide B (Fermi enhanced overtone of amide II band) -	Protein
3	2929	2925	2926	ν_{as} CH_2	
4		2852	2854	ν_s CH_2	
5		1744		ν C=O (Ester)	polysaccharide
6	1651	1652		Amide I – Protein α Helix	Protein
7			1624	Amide I	Protein
8	1535	1535	1530	Amide II	Protein
9	1456	1456	1456	ν_s C=O of COO^{-} δ_s CH_3 of methyl groups of protein	Protein
10			1412	νC-N , δ N-H, δ C-H	Protein
11	1385	1381	1385	δ_s CH_3, νC-O δ C-H, δ N-H	
12			1339	ν C-O in plane + ring stretch phenyl.	
13			1215	Amide III – Protein	Protein
14			1161	ν C-O / C-OH vibration	polysaccharide
15	1107			ν C-O, ν C-C ring	polysaccharide
16			1038	ν C-C, (ν CH_2OH), ν C-O+ δ C-O , ν S-O Cysteine monoxide	
17	1022	1022		δ C-O , δ C-C, δ O-CH, ring	polysaccharide
18	991	994		ν C-O , ν C-C Vibration	
19			972 shoulder	ν_{as} N-CH_3,O-CH_3	polysaccharide
20	906	907		Phosphodiester stretching region	polysaccharide
21		881	882	C-O, C-O vibration	
22	794	793		CH out of plane bending	
23	749	750		CH out of plane bending	
24			719	C-H vibration > CH_2, N–H Amide IV	Protein
25	680	679	669	CH out of plane bending vibration	
26	638			OH out of plane bending, -CH out of plane bending	
27	520	520	518	Cα = Cα torsion and ring ring torsion of Phenyl (1)	

ν – stretching vibration; δ – bending vibration; s - symmetric; as- asymmetric

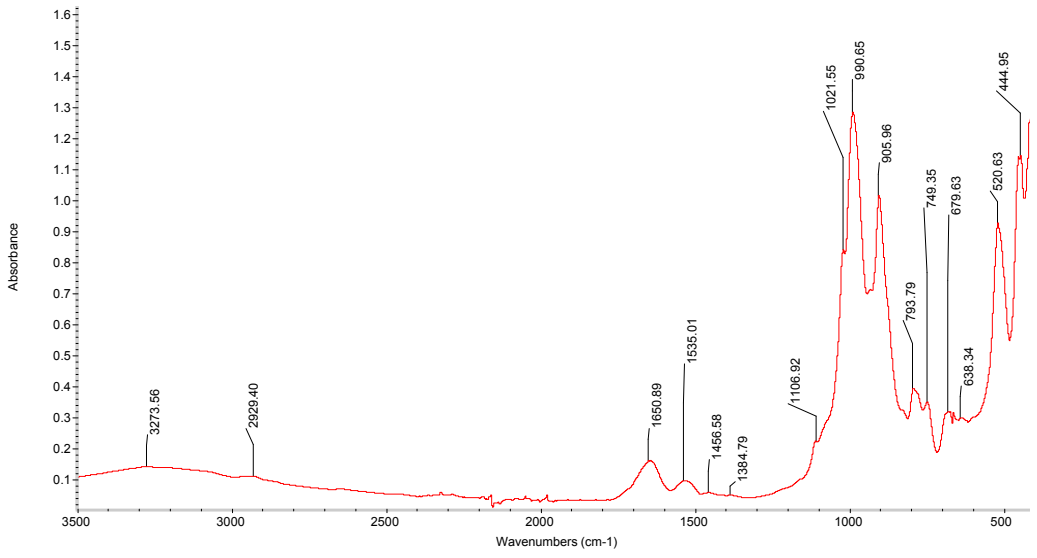

Fig. 1.FTIR spectra of biofilm EPS of S. agalactiaeSA3 grown on 0.625 % LB glucose with 0.3% bentonite clay

FT-IR analysis of *S. agalactiae*free cell components

The major peaks identified and characterized in free cell components of *S. agalactiae*were at wavenumber3273cm^{-1} (amide A, N-H stretching,protein),3084 cm^{-1} (amide B, N-H stretching,protein) 1624cm^{-1} (Amide I β pleated sheet in protein.), 1530cm^{-1}(amide IIin protein),

1530 cm^{-1}, 1456 cm^{-1} and 1215 cm^{-1} and werepredominant in free cell components. Two peaks specific for proteins (1412 cm^{-1} and719 cm^{-1}) were weak, three peaks specific for polysaccharides(1161 cm^{-1}, 1038 cm^{-1} and 972 cm^{-1})and were very weak in free cell components(Fig.3. andTable1).

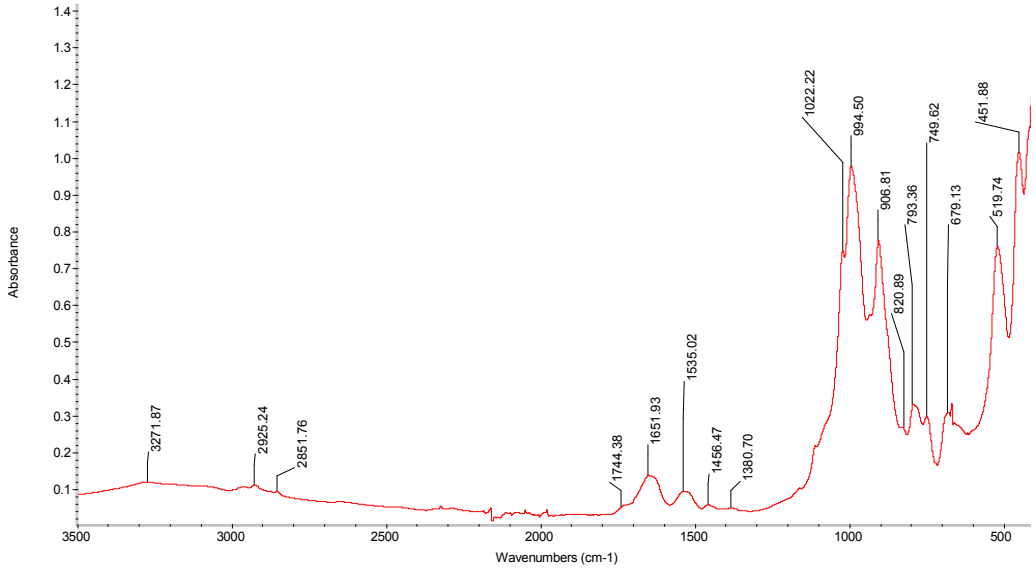

Fig. 2. FTIR spectra of biofilm EPS of S. agalactiaeSA3 grown on 2.5 % LB glucose on glass beads

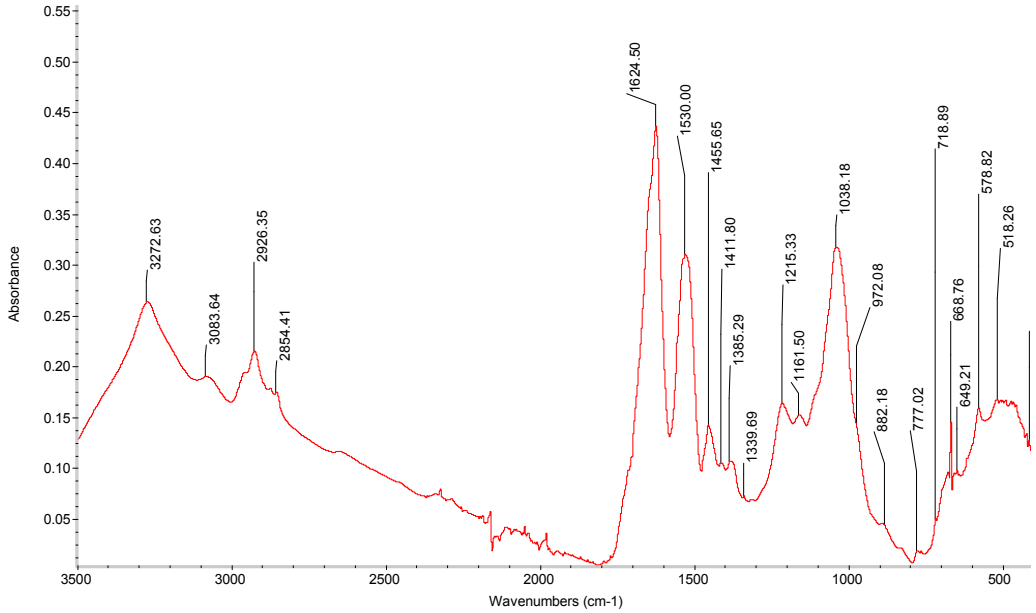

Fig. 3. FTIR spectra of free cells components of S. agalactiaeSA3 grown on 2.5 % LB glucose

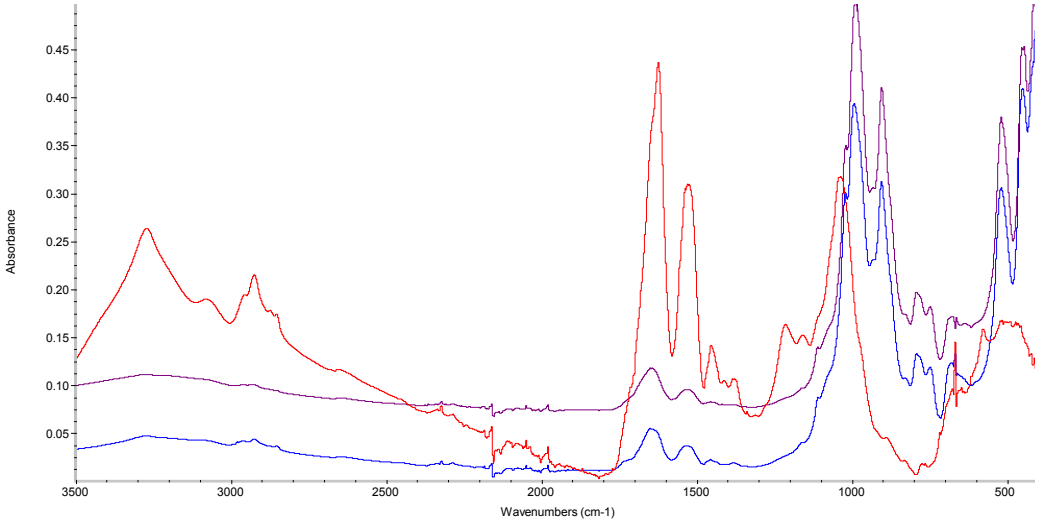

Fig. 4. Composite FTIR spectra of biofilm EPS of S. agalactiaeSA3 grown on bentonite clay and glass beads and free cells components

DISCUSSION

Mastitis is a complex disease, with multiple etiological factors, different degrees of intensity, variations in duration and residual effects. Mastitis remains the most common disease of dairy cattle and many producers continue to struggle to achieve the production of quality milk. Mastitis results when pathogenic bacteria are able to gain entrance in to the udder, overcome the animal immune defenses, establish an infection and produce inflammation of udder secretary tissue.Among the mastitis causing bacterial agents, *S. agalactiae* is one of the most predominant pathogens causing clinical mastitis. In recent days, the number of mastitis cases not responding to generally used antibiotics therapy are increasing, this could be attributed to frequent and indiscriminate use of antibiotics, emergence of drug resistant *S. agalactiae* strains besides the potential biofilm forming ability of this organism. The contribution of biofilm forming ability to complexity of such bacterial infection has been extensively studied (Costerton *et al.,* 1995 and Mah *et al.,* 2001)and one of the most convincing hypothesis to explain therapeutic resistance is the ability of many bacterial infections to grow as biofilm in infected tissues, thus developing an innate resistance to almost all therapeutic agents.

Analysis of *S. agalactiae* biofilm exopolysacharides and free cell components

Exopolysaccharides (EPS) in biofilms are important in the attachment of bacteria to substrata and thus development of biofilms (Costerton *et al.,* 1987).Exopolysaccharides are excreted from multiple bacterial species, which make biofilms, a good source for screening EPS producing bacteria (Davey and O'Toole, 2000). During the process of colonization on particular surfaces, bacteria produces extracellular polymeric substance which constitutes the biofilm matrix (Geesey and White, 1990).These polymeric substances mainly comprised of EPS (40-95%), protein (1-60%), lipids (1-40%) and nucleic acids (1-10%) (Davey and O'Toole, 2000; Flemming and Wingender, 2001). Microbial cells generally contain various polysaccharide structures contributing to their shape and rigidity. Capsular EPS are produced mainly during the log phase of bacterial growth and slime EPS produced during the stationary phase (Plante and Shriver.1998). In the present study, an attempt was made to analyse the *S. agalactiae* biofilm EPSin comparison with free cell components by FTIR spectroscopy.

FT-IR analysis of EPS of *S. agalactiae*biofilms and free cell components

The infrared spectroscopic fingerprints of EPS of biofilms and free cell components show both major and subtle chemical compositional changes between the biofilm EPS and free cell components. In the EPS of *S. agalactiae* biofilms grown on bentonite clay, the FT-IR spectra indicated the increasing IR signalling intensities at the polysaccharide spectral regions with the major peaks of polysaccharides at 1107 cm^{-1}, 1022 cm^{-1}, 991 cm^{-1} and 906 cm^{-1} wavenumbers. Among these, polysaccharidepeaks at 1022 cm^{-1}, 991 cm^{-1} and 906 cm^{-1} wavenumber were more prominent indicating their higher concentrations in biofilm EPS, where as the polysaccharide peak at 1107 cm^{-1} wavenumber was not prominent indicating its lower concentration in biofilm EPS. The decreasing IR signalling intensities in the protein spectral region with the peaks at 3274 cm^{-1}, 1651 cm^{-1}, 1535 cm^{-1} and 1456 cm^{-1}wavenumbers specific for proteins were not prominent indicating lower concentration of proteins in biofilm EPS (Fig.1 and Table 1).The FT-IR spectra of the EPS of *S. agalactiae* biofilms grown on glass beads indicated the increasing IR signalling intensities at the polysaccharide spectral regions with the major peaks of polysaccharides at 1022 cm^{-1}, 994 cm^{-1} and 907 cm^{-1} wavenumbers were very predominant in biofilms grown under this particular condition. The decreasing IR signalling intensities in the protein spectral region with the peaks at 3272 cm^{-1},1652 cm^{-1}, 1535 $cm^{-1}$and 1456 cm^{-1} wavenumbers specific for proteins were not prominent in EPS of biofilm grown on glass beads indicating lower concentration of proteins in biofilm EPS (Fig. 2 and Table 1).In the *S. agalactiae* free cell components, the FT-IR spectra indicated the increasing IR signalling intensities at the protein spectral regions with the major peaks at 3273 cm^{-1}, 3084 cm^{-1}, 1624 cm^{-1}, 1530 cm^{-1}, 1456 cm^{-1} and 1215 cm^{-1} wavenumbers specific for proteins which werepredominant in free cell components indicating the higher concentration of proteins in the free cell components. Whereas the decreasing IR signalling intensities in the protein spectral region with two protein specific peaks at 1412 cm^{-1} and719 cm^{-1} wavenumbers which were not prominent indicating their lower concentration.It was also noted that the decreasing IR signalling intensities in the polysaccharide spectral region with three peaks at 1161 cm^{-1}, 1038 cm^{-1} and 972 cm^{-1} wavenumbers which were specific for polysaccharides were not prominent indicating their lower concentration of polysaccharides in free cell components (Fig. 3 and Table 1).

Bacterial biofilms are composed primarily of microbial cells and EPS. Exopolysaccharide substances may account for 50 to 90 per cent of total organic carbon of biofilms and can be considered the primary matrix material of the biofilms.Exopolysaccharide substances may vary in chemical and physical properties, but primarily it is composed of polysaccharides (Ward *et al.,* 1992).Many researchers have also studied the FT-IR spectra of EPS matrix of various bacterial biofilms. Diego *et al.* (2008) studied the difference between the biofilm and planktonic cells of *Bordetella pertussis* (*B. pertussis*) using FT-IR spectroscopy and reported that the distinctiveness of the *B. pertussis* biofilm polysaccharide polymeric matrix over their planktonic counter parts. Similarly, Yongqin*et al.(*2010) studied and compared the composition of EPS of acidophilic microbial biofilms from mines using FTIR spectroscopy and reported that more than twice as much EPS was derived from mature biofilms as from immature biofilms. The EPS composition analyses indicated the presence of carbohydrates, metals, proteins, and minor quantities of DNA and lipids.

In the current study, FTIR spectra has shown marked differences in the chemical composition of the biofilm EPS and free cell components. Differences were observed in the composition of the biofilm EPS and free cell components particularly in the carbohydrate region between 1200-900 cm^{-1}.Interestingly, the *S. agalactiae* biofilms grown on bentonite clay and glass beads showed increasing IR signalling intensities at the polysaccharide spectral regions, instead, the free cells showed decreasing IR signalling intensities at this region. These findings clearly indicated that the polysaccharide concentrations are more in *S. agalactiae*biofilms compared to

free cell components. These temporal differences reflect the presence of excess amount of polysaccharides in biofilms and may be related to exopolysacharides production during biofilm development. These findings were in conformity with Schmitt *et al.* (1998) and Michele. (2009),who also demonstrated FTIR spectral region of *Pseudomonasaeruginosa* biofilms and showed that theintensity and composition of bands at 1200 cm^{-1} and 900 cm^{-1} vary considerably during the biofilm formation and these regions are mainly correlated with EPS.The differences were also observed in the composition of the biofilm EPS and free cell components particularly in the protein region. The biofilms grown on bentonite clay and glass beads showed decreasing IR signalling intensities at the protein spectral regions, Instead, the free cells showed increasing IR signalling intensities at protein region, indicating the presence of higher concentration of proteins in the free cell components, which might be because of the release of cell associated proteins during the process of extraction, as the same method of EPS extraction was applied for free cell components also. As a result it was found that very less amount of polysaccharides in the free cell pellet that might be capsular polysaccharides and/or cell associated polysaccharides and not the one which were present in case of biofilm EPS.

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REFERENCES

Cira, D. R., Roberto, R., Cira, L, G., Francesco, B., Francesca, N., Valerio, R., Claudia, G., John, L, T., Guido, G. and Domenico, M. 2010. Specific involvement of pilus type 2a in biofilm formation in group B streptococcus. *PLoS one.,*5: 1-11

Costerton, J. W., Cheng, K. J., Geesey, G. G., Ladd, T. I. Nickel, J. C., Dasgupta, M. and Marrie, T. J. 1987. Bacterial biofilms in nature and disease. *Annu. Rev. Microbiol*.,41: 435–464.

Costerton, J. W., Lewandowski, Z., Caldwell, D, E., Korber, D, R. and Lappin, S, H. 1995. Microbial biofilms. *Ann. Rev. Microbiol.,*49: 711–745

Costerton, J. W., Stewart, P. S. and Greenberg, E. P. 1999. Bacterial biofilms: a common cause of persistent infections. *Science.*, 284: 318–322

Davey, M. E. and O'Toole, G. A. 2000. Microbial biofilm from ecology to molecular genetics. *Micrbiol.MolBiol.Rev.,*64: 847-867

Diego, O. S., Genia, L., Florian, W., Stefan, S., Angelika, G., Osvaldo, M.Y. and Monika, E. 2008. Proteome approaches combined with Fourier transform infrared spectroscopy revealed a distinctive biofilm physiology in *Bordetella pertussis*. *Proteomics.*, 8: 4995–5010

Flemming, H. C. and wingender, J. 2001. Relevance of microbial extracellular polymeric substances (EPS)- Part I: *Structural and Ecological aspects Water Sci.Tech.*, 43:1-8

Geesey, G. G. and White, D. C. 1990. Determination of bacterial growth andactivity at solid-liquid interfaces. *Ann.Rev.Microbiol.*, 44: 579-602

Ghiorghi, Y. K., Emilie, M., Adeline, M., Guillaume, D., Elise, C., Patrick, T. and Shaynoor, D. 2009. Dual role for Pilus in adherence to epithelial cells and biofilm formation in *Streptococcus agalactiae*. *PLoS. Pathog.,*5: 422

Goldberg, J. J., Pankey, J. W., Drechsler, P. A., Murdough, P. A. and Howard, D. B. 1991. An update survey of bulk tank milk quality in Vermont. *J. Food Protect*., 54: 549-553

Kirti, J. 2011. Studies on isolation and characterization of oral biofilm forming bacteria. Masters Thesis, National Institute of Technology, Rourkela, Odisha, India

Mah, T. F., and O'Toole, G, A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol*., 9: 34–39

Merle, E. O., Howard C., Douglas, W. M., Andre, G. B. and Ronald, R. R. 2002. Biofilm bacteria: formation and comparative susceptibility to antibiotics.,*Can. J. of Vet.* Res., 66: 86-92

Michele, Y. M. S. 2009. Investigation of Pseudomonasbiofilm development and removal on dairy processing equipment surfaces using Fourier transform infrared (FT-IR) spectroscopy. *Ph.D. thesis* the Ohio State University, USA.

Movasaghi, Z., Rehman, S. and Rehman, I. 2008. Fourier transform infrared (FTIR) spectroscopy of Biological tissue. *App. SpectroscpyReviews.,*43:134- 179

Muthusamy, A. K., Kanapathi, T. A. and Karuppiah, P. 2011. Production and characterization of exopolysaccharides (EPS) from biofilm forming marine bacterium. *Braz. arch. biol. technol.,*l54 (2): 259-265

Nasim, M. 2012. Detection of biofilm formation ability of *Streptococcus agalactiae* isolated form bovine mastitis. M.V.Sc. Thesis, Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, India.

Naumann, D., Fabian, H. and LASCH, P. 2009. Fourier transforms infrared (FTIR) spectroscopy of cells, tissue and body fluids in biological and biochemical Infrared spectroscopy. *Eds-barth A, Haris P. I , ISO press*.PP 312-354.

Plante, C. J. and Shriver, 1998. Differential lysis of sedimentary bacteria by Arenicola marina L: examination of cell wall structure and exopolymeric capsules as correlates., *J. Exp. Mar. Biol. Ecol.*, 229:35-52.

Schmitt, J. and Flemming, H. C. 1998. FTIR-spectroscopy in microbial and material analysis. *Int. Biodeterior. Biodegradation.,*41: 1-11.

Schoonderwoerd, M., Rawluk, S., Ollis, G. and Schipper, C. 1993. Prevalence of *Streptococcus agalactiae* in Alberta dairy herds. Farming for the Future, Alberta agriculture food and rural development.

Sheetal, P. 2011. Isolation, characterization and stress response of dental plaque forming bacteria. Masters Thesis, National Institute of Technology, Rourkela, Odisha, India

Stuart, B. 1997. Biological application of Infrared spectroscopy, ACOL Seriesmwiley, Chichester, UK

Sutherland, I. 2001. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiol.,*147: 3–9.

Ward, K.H., Olson, M. E., Lam, K. and Costerton, J.W. 1992. Mechanism of persistent infection associated with peritoneal implants. *J. Med. Micro.,*36:406-413.

Yongqin, J., George, D. C., Anna, K. H., Paul, W., Matthew, S., Korin, E. W., Jillian, F. B. and Michael, P. T. 2010. Characterization of extracellular polymeric substances from acidophilic microbial biofilms. *Appl. Environ. Microbiol.*, 76: 2916–2922.

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