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RESEARCH ARTICLE

Comparison of Planktonic and Sessile Cells of *Cronobacter sakazakii*

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Abstract

Cronobacter sakazakii is an emerging food borne pathogen that causes severe meningitis, meningoencephalitis, sepsis, and necrotizing enterocolitis in neonates and infants, with a high fatality rate. It has been known that the pathogen forms biofilms on latex, polycarbonate, silicon rubber and glass. The present study aims to analyze and compare the biofilms of the standard *Cronobacter sakazakii* (MTCC-2958) and an isolate (Jal1) from goat milk sample employing Scanning Electron Microscopy (SEM). The revived cultures were streaked on to the Tryptic Soya Broth plates with 1% glucose and sterilized substrates. The biofilms formed on the surface of the substrates were then fixed by modified Karnovsky's method. Substrates were sputter coated and analyzed by SEM. Biofilms formed on steel and glass substrates but failed to form on the aluminium substrate. Moreover the biofilms formed by isolate 'Jal1' on glass and steel was denser than that formed by standard *Cronobacter sakazakii* (MTCC-2958).

Keywords: Biofilms, *Cronobacter sakazakii,* Planktonic, Scanning electron microscopy

Introduction

Planktonic or sessile cells are the two distinct states in which bacteria generally exist in nature. Planktonic cells are classically defined as free flowing bacteria in suspension' as opposed to the sessile biofilm state: 'a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton *et al*., 1999). They may also be found in chronic infections as suspended in host materials (Burmolle *et al*., 2010).

Glycocalyx (Costerton *et al*., 1981) is a matrix (synthesized by the bacteria) containing extracellular fibrous polysaccharide and is very important for biofilm formation as it helps in the adherence of biofilms of bacterial cells permanently to the substratum (Fletcher and Floodgate, 1973), and also offers structural stability and increased tolerance to antimicrobial and immune cells (Mulcahy *et al*., 2008; Ma *et al*., 2009).

Biofilms may form in varying environments and surfaces when supplied with moisture and nutrients. They are usually composed of polysaccharides, proteins, nucleic acids and cell components with a high concentration of minerals and nutrients from the surrounding environment (Sutherland, 2001; Dunne, 2002; Allison, 2003).

Biofilms grow on the contact surface with moisture and nutrients and have been reported to occur in nature as well as in food and medical industries. Formation of biofilms contribute to serious problem as they cause severe damage to various equipments, contaminate surface of food and various processing systems (Kumar and Anand, 1998; Poulsen, 1999).

Cronobacter sakazakii (MTCC-2958), an opportunistic pathogen (Johler *et al*., 2010; Hartmann *et al*., 2010), belongs to the family Enterobacteriaceae, earlier known as *Enterobacter sakazakii* (Iversen *et al*., 2008). It is a Gram-negative, motile, rod shaped, non-spore-forming bacterium which can grow in both aerobic and anaerobic conditions. It is found in neonates, first reported by Urmenyi and Franklin (1961), and causes meningitis (inflammation of the protective membranes covering the brain and spinal cord) and sepsis (whole-body inflammatory state called asystemic inflammatory response syndrome or SIRS) that is triggered by an infection and enteritis(inflammation of the small intestine) among the neonates(Acker *et al*., 2001; Hunter *et al*., 2008; Muytjens *et al*., 1983; Biering *et al*., 1989; Bar-Oz *et al*., 2001). It has been known that the pathogen forms biofilms on latex, polycarbonate, silicon rubber and glass (Iversen *et al*., 2004; Lehner *et al*., 2005).

E. sakazakii infections in infants and immune-compromised adults are caused by foods such as powdered infant formula and fresh produce, respectively. Contact of the pathogen, *E. sakazakii,* with any of the food in abiotic or biotic surfaces may result in the formation of

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biofilms especially on equipment surfaces used in formula preparation and feeding areas or in produce processing plants. The surfaces such as spoon, brush, and blender used for infant formula preparation are found to be prone to the *E. sakazakii* colonization (Bar-Oz *et al*., 2001; Muytjens *et al*., 1983; Noriega *et al*., 1990; Acker *et al*., 2001) and also it has been reported that re-use of enteral feeding tubes and delivery bags after washing has greater risk of infection of the microbes (Robbins *et al*., 2005). The fruits and vegetables during harvesting, transporting, processing, and storage have been encountered to form biofilms and potentially increase the risk of diseases in individuals consuming these products (Bar-Oz *et al*., 2001). It was observed by Kim *et al*., 2006 that *E. sakazakii* is able to grow on fresh-cut fruit and in its juice. Scheepe-Leberkuhne and Wagner (1986) have reported that *E. sakazakii*, produce viscous capsular material, and therefore the organism could form a biofilm on feeding equipment and contact surfaces.

Proteomic profiles of biofilm and planktonic cells were studied by Yingwang et *al*., 2016 which significantly revealed 28 differentially expressed proteins between biofilms and planktonic cells. PPIase (Peptidyl-prolyl isomerase), FlgE and DsbC were found expressed in only sessile cells while other proteins such as LuxS, ompC, TolB, Mg1B activity increased in biofilm cells. Jielin *et al*., 2016 provided data regarding the cellular requirements for the cells to undergo biofilm formation and survival in extreme conditions and accordingly expression of about 1190 proteins related to biological binding, cell structure, signal transduction, cell adhesion and cellular interaction were increased.

Materials and Methods

Isolation and identification of bacterial strain

The isolate (Jal1) from goat milk sample, under study was procured from Cell Biotechnology Lab, DEI, Agra (Sharma and Prakash, 2014a). Standard strain of *Enterobacter sakazakii* (MTCC-2958) was used as positive control. EE (*Enterobacter* enrichment) broth was used for enrichment after 24 h of incubation.

The reference strain (MTCC-2958) and Jal1 were biochemically characterized through 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, and a sugar fermentation tests.

The isolate was confirmed to be *C.sakazakii* as per Sharma and Prakash (2014b) by molecular characterization based on *ompA* gene specific to *C.sakazakii*.

Harvesting biofilms of the type strain (MTCC- 2958) and Jal

The revived cultures were streaked on Trptone Soya Agar (TSA) plates. An isolated colony from TSA was grown at 37^0C for 24h in 10ml Tryptic Soya Broth (TSB) with 1% glucose (Bose *et al*., 2009) and adjusted to cell density of 10^8 CFU ml^{-1} using Mc Farland standard along with sterilized substrates such as glass, steel coupons and aluminium to compare the adherence of biofilms on each substratum. These substrates were selected because they are commonly used.

Fixation of Biofilms on Substrates

Incubation of cultures in TSB for 24 h led to formation of biofilms on various substrates. The broth was decanted and the substrata with biofilms were washed with PBS buffer (Phosphate Buffered Saline- 1.4g 0.2M-Disodium hydrogen phosphate, 1.56g 0.2M-Sodium dihydrogen phosphate and 0.4g 0.8%(w/v)-NaCl in 100ml water; pH 7.3) After washing, the substrates were dried and were fixed in fixing solution as per the modified Karnovsky's method (Oliveira *et al*., 2010).

Later, substrates were incubated for 24h in 0.05M sodium cacodylate buffer at pH 7.2 containing 0.001M calcium chloride. After incubation, the substrates were washed thrice with 0.05M sodium cacodylate buffer at pH 7.2 for about 10 min. Fixation was done in osmium tetraoxide (1% in distilled water) for 1h at ambient temperature and then again washed thrice with distilled water. The biofilms on substrates were dehydrated in acetone dilutions of 25%, 50%, 75%, 90% and 100% for 2-3 min in each. Washing with each dilution was done thrice. The substrates were then transferred to the critical point apparatus for complete drying. Each substratum was sputter coated with gold. The samples were subjected to scanning electron microscopy (SEM) for comparison of the biofilms.

Preparation of planktonic cells for SEM

The enriched cultures of the reference strain (MTCC- 2958) and Jal1 (10^8 CFU ml^{-1}) were centrifuged at 6000 rpm for 10 min. The supernatant was discarded and pellet obtained was washed with PBS buffer. This was again centrifuged for 5min at 6000 rpm. The supernatant was discarded and the pellet was obtained. The pellet was used to make a smear on the slide and was allowed to dry for the fixation process. Fixation was carried out in the same manner as that for biofilms. These arrested cells were then subjected to SEM.

Results and Discussion

Results in Table 1 depict biochemical characterization of the isolate. MTCC-2958 was taken as a positive control for all the biochemical tests performed. Fermentation tests

revealed Jal1 and MTCC-2958 fermented glucose, maltose but not arabinose (Table-1)

Table 1 : Biochemical characterization of Reference MTCC-2958 and the Isolate

Biochemical Tests		Results	
		MTCC-2985	Jal1
Methyl Red		Negative	Negative
Voges Proskeur		Positive	Positive
Indole		Negative	Negative
Nitrate		Positive	Positive
Sugars			
	a. GLUCOSE	Positive	Positive
	b. MALTOSE	Positive	Positive
	c. ARABINOSE	Negative	Negative
Simmon Citrate		Positive	Positive
TSI			
	1. Slant	Negative	Negative
	2. Butt	Positive	Positive
	3. Bubble	Positive	Positive
Catalase		Positive	Positive
Oxidase		Negative	Negative

Comparison of the biofilms of MTCC-2958 and the isolate, on different substrata (steel, glass and aluminium) and planktonic cells was done by scanning electron microscopy.

Scanning Electron Micrographs (Fig. 1 & 2) depict the growth of biofilms formed on steel by MTCC-2958 cells and Jal1 respectively. On comparison it was observed that the growth of the latter was denser than that of the former. Whereas it was observed in case of glass substrata that the MTCC-2958 cells (Fig. 3) adhered more to the glass substratum as compared to the cells Jal1 (Fig. 4).

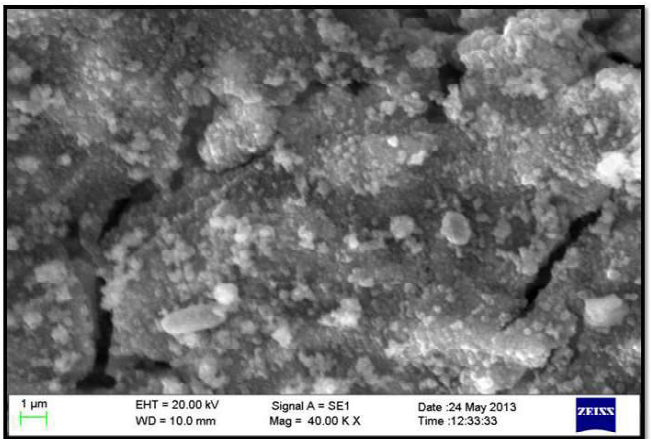

Fig.1. Scanning electron microscope micrograph of biofilms of the reference *Cronobacter sakazakii* MTCC-2958 grown on steel substratum at 40KX magnification

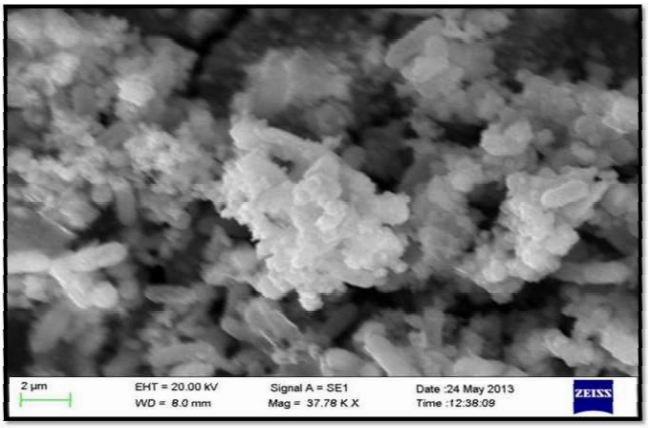

Fig.2. Scanning electron microscope of biofilms of Jal1, grown on steel substratum at 37.78KX magnification

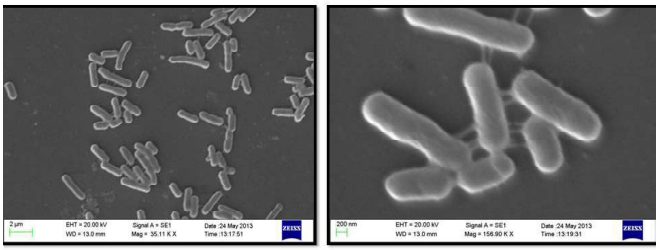

Fig.3. Scanning electron microscope micrograph of the reference *Cronobacter sakazakii* MTCC-2958 grown on steel substratum (a) at 35.11KX magnification (b) at 156.90KX magnification showing adhesion threads

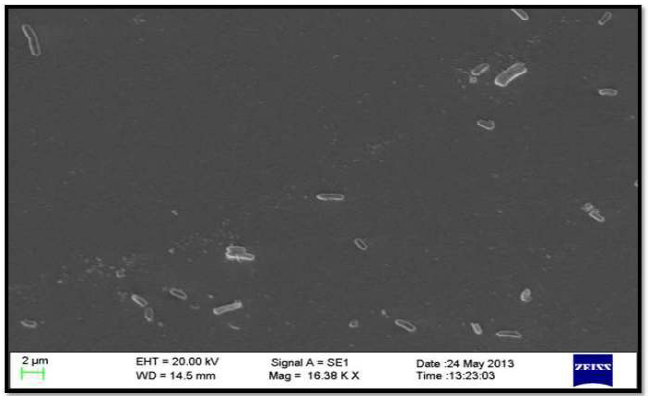

Fig.4. Scanning electron microscope micrograph of biofilms of Jal1, grown on glass substratum at 16.38KX magnification

Interestingly no growth was observed for both the biofilm cells of the MTCC 2958 cells (Fig. 5) and Jal1, on the aluminium substrate (Fig. 6).

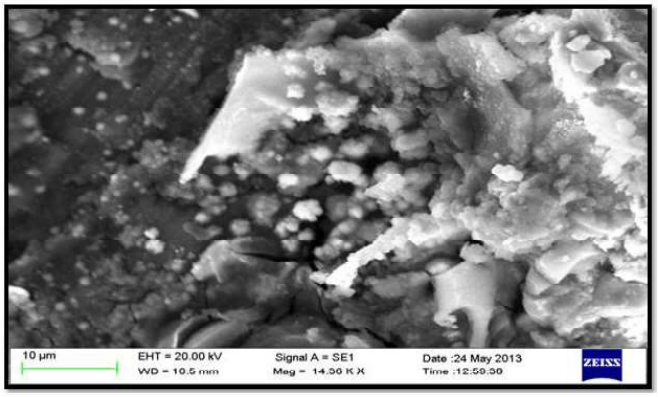

Fig.5. Scanning electron microscope micrograph of biofilms of the reference *Cronobacter sakazakii* (MTCC-2958) grown on aluminium substratum at 4.36KX magnification

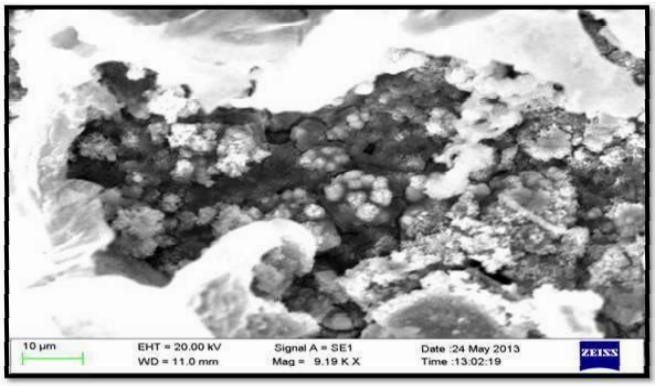

Fig.6. Scanning electron microscope micrograph biofilms of Jal1, grown on aluminium substratum at 9.19KX magnification

The SEM micrograph of planktonic cells of MTCC-2958 cells and their dimensions are shown in Fig. 7.

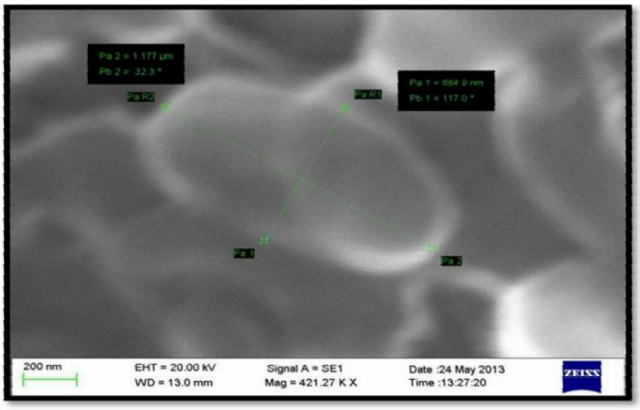

Fig.7. Scanning electron microscope of planktonic cell of the reference *Cronobacter sakazakii* (MTCC-2958) at 421.27 KX magnification and its dimensions

From the present findings it was observed that the adherence of biofilms formed by MTCC-2958 cells on the surface of steel is less dense as compared to the biofilms of Jal1 (Fig. 1 and 2). A large number of clumps of the biofilms of the isolate were observed in the SEM micrograph. Although no reports have been reported in reference to this observation. The formation of biofilms of Jal1 therefore shows a thicker growth. Chemical differences between planktonic and sessile cells of *C.sakazakii* have been clearly elaborated through FTIR and Raman spectroscopy (Sharma and Prakash, 2014).

Aluminium is the third most abundant metal which is extensively used for utensils in homes since ancient times. It is a highly reactive metal which reacts more readily with oxygen to form aluminium oxides. The aluminium oxide thus formed is a protective layer on the surface which helps in prevention of corrosion of the metal. The biofilms formed on the surface of aluminium MTCC-2958 cells and the isolate did not adhere to aluminium. This could be supported by the fact that the formation of aluminium oxide on the surface may be hindering the pathogen to form adhesion threads over the surface (Fig. 5 and 6).

From the experiment it was concluded that biofilms are found to be more adherent to glass surface. Fig. 3 shows the biofilms of *C.sakazakii* (MTCC-2958) on glass substrata. From the figure it can be observed that pathogenic cells form adherence threads with the surface that leads to the formation of small colonies and subsequently the mature ones. This can be of concern as glass has become a popular domestic and industrial material of use over the years. FTIR-spectra depicted the shift in functional groups related to carbohydrates and proteins on comparing the planktonic and biofilm cells (Schmitt and Fleming 1998; Bosch *et al*., 2006; Mukherjee *et al*., 2011). Virulence characteristics of the biofilms are contributed by an increase in the production of uronic acid in EPS (Bosch *et al*., 2006). Uronic acid, an acid sugar, may help the stabilization of glycosidic linkages with the help of the carboxylic acid moiety and thus sessile cells attain higher resistance to acid hydrolysis (Fett *et al*., 1995). On comparison of the biofilm formation of Jal1 (Fig. 4) to that of the MTCC-2958, it was observed that the former had a larger number of biofilm cells in clumps.

In the present research, increase or decrease in biofilm cells on different substrates (glass, steel, and aluminium) can be supported with difference in expression profile of proteins estimated through FTIR and Raman Spectroscopy (Sharma and Prakash 2014). In addition to polysaccharides, proteins (Gerlach and Hensel, 2007) and lipopolysaccharides (Donlan and Costenton, 2002; Hall-Stoodly and Stoodly, 2002) present in EPS may aid in

initial attachment of bacteria to abiotic surfaces and thus help in biofilm formation.

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