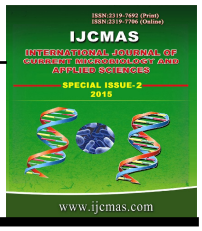
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Special Issue-2 (May-2015) pp. 38-47**
http://www.ijcmas.com



Original Research Article

Isolation and characterization of biofilm forming *Streptococcus* species from oral flora of cancer patients

Anjali T. Jadhav* and Vidya S. Tale

Department of Microbial Biotechnology, Rajiv Gandhi Institute of IT and Biotechnology, Bharati Vidyapeeth Deemed University, Pune 411046, India

**Corresponding author*

Keywords

Biofilm, Hemolysis, *Streptococcus* sp.

A B S T R A C T

A biofilm is a complex aggregation of microorganisms growing on a solid substrate. It acts as a defensive tool for microorganisms during various stress conditions. The proposed work was carried out to study the biofilm forming ability of microorganisms specifically *Streptococcus* sp. from oral samples of cancer patients. The bacteria was isolated using Mitis Salivarius medium which is selective for growth of *Streptococcus sp*. Morphological and biochemical characterization of isolates were carried out. Glucan synthesis was observed on Mitis Salivarius medium. All three isolates were found to be Gram positive chains resembling like *Streptococci*. Biochemical identification was performed. Catalase test was negative for all the isolates. Sugar fermentation using different sugars like mannitol, lactose and ribose indicated only acid production. Hemolytic properties of isolates were also studied using Blood agar medium, indicated α hemolysis and γ hemolysis. Biofilm forming ability was studied by congo red agar method (CRA), tube assay and microtitre plate assay. Optical density values at 570 nm were considered as an index of attachment to the surface for formation of biofilm. Various anti-biofilm agents will be further screened for the anti-biofilm potential.

Introduction

A biofilm is complex aggregation of microorganisms growing on a solid substrate. These adherent cells are embedded within self-produced matrix of extracellular polymeric substance (EPS). Biofilms grow in the three stage process. Initially they get attached to the substratum, bacterial growth and division leads to colonization of the surrounding area and the formation of biofilms.

Mature biofilms are a complex diverse structure of dormant and actively growing bacterial colonies along with enzymes and excretory products. The major features that distinguish biofilm forming bacteria from planktonic counterparts are surface attachments ability, high population density, extracellular polymeric substances (Beer and Stoodley, 2006). Oral microbial-plaque is the community of microorganisms found on

a tooth surface as a biofilm, embedded in a matrix of polymers of host and bacterial origin (Marsh, 2004). Bacterial surface proteins which bind to host extracellular matrix proteins often play a key role in initial adherence of bacteria to solid surfaces within the host (Patti *et al.,* 1994). EPS production is known to be affected by nutrient status of the growth medium. EPS is distinct both chemically and physically from the bacterial capsule (Mckenney, 1998).

Different organisms produce differing amounts of EPS and the amount of EPS increases with age of the biofilm. Formation of biofilm may result in diseases including native valve endocarditis, osteomyelitis, dental caries, middle ear infections, medical device-related infections, ocular implant infections and chronic lung infections in cystic fibrosis patients (Donlan and Costerton, 2002). Biofilm is also responsible for the formation of oral diseases such as dental caries and plaques. These oral diseases affect the majority of population throughout the world (Marsh, 2006). Biofilm formation acts as a defensive tool during various stress conditions (Patel, 2005). Microorganisms within biofilm can withstand nutrient deprivation, pH changes, oxygen radicals, disinfectants and antibiotics better than planktonic microorganisms (Marsh, 2003). Biofilms are also resistant to phagocytosis and these phagocytes which attempt and assault on the biofilm may harm more to the surrounding tissue rather than to the biofilm, making biofilms extremely difficult to eradicate from living hosts (Lewis, 2001). *Streptococci, Actinomyces* and *Lactobacilli* are major initial colonizers on the tooth surface. *Streptococcus sp.* has the ability to metabolize various carbohydrates into organic acids which may lead to the cariogenic destruction of tooth surfaces.

Proposed work is based on the study of biofilm forming bacteria and their characterization.

Materials and Methods

Isolation of *Streptococcus sp.* from oral samples

Oral swabs were collected from cancer patients and inoculated in Mitis Salivarius (MS) broth medium [Casein enzymatic hydrolysate (15 g/lit), peptic digest of animal tissue (5 g/lit), dextrose (1 g/lit), sucrose (50 g/lit), dipotassium phosphate (4 g/lit), trypan blue (0.075 ml/lit), crystal violet (0.0008 g/lit),pH= 7.0] for the isolation of *Streptococcus sp.* Inoculated MS broth was incubated at 37^0 C for 24 hours. MS broth culture after incubation was further transferred on MS agar medium and incubated at 37^0 C for 24 hours. Colony characteristics of randomly isolated colonies were recorded. Gram staining was performed.

Biochemical characterization

Selected colonies were further characterized for various biochemical tests including catalase activity, sugar fermentation test. Hemolysis pattern was observed on blood agar supplemented with 5% sheep blood. Colonies were streaked on blood agar and incubated for 24 hours at 37^0 C (Aneja, 2003).

Screening of isolates for biofilm formation

Congo red agar method (CRA)

Biofilm formation by the isolates was checked by using CRA medium (Freeman *et al.,* 1989). Sterile CRA plates were inoculated and incubated at 37^0 C for 24 hours. Black colonies with dry crystalline consistency were considered to be positive for biofilm production.

Microscopic observation of biofilm

Microscopic observation of biofilm was done using tissue culture plates. Overnight grown cultures of isolated *Streptococcus sp.* were inoculated in Trypticase soy broth (TSB) and incubated at 37^0 C for 24 hours without shaking. Plates were washed with Phosphate Buffered Saline (PBS, pH 7.3) and stained with 0.1 % crystal violet for 30 minutes and again washed with 95 % ethanol and dried.

Biofilm formation assay

Tube assay

Biofilm formation was also checked by the tube assay. TSB medium (5 ml) supplemented with 5% sucrose was inoculated with 100 µl of overnight grown culture broth and incubated for 24 hours at 37^0 C. The tubes were decanted and washed with phosphate buffer saline (PBS) (pH 7.3). Two sets were prepared, one kept in static condition and another was kept at shaking condition for 24 hours.

Staining of tubes was done by 0.1 % crystal violet. Excess stain was removed by washing the tubes with 95 % ethanol and dried.

Formation of biofilm was confirmed with the presence of attachment (visible film) on the wall and bottom of tube (Christensen *et al.*, 1982).

Microtitre plate assay

All the isolates were screened for their biofilm forming ability by using microtitre plate method. Overnight grown culture (200 µl) was inoculated in TSB media supplemented with 5% sucrose and incubated in microtitre plate at 37^0 C for 24 hours. Contents of each well were gently removed and wells were then washed with PBS (pH 7.3) to remove free floating planktonic bacteria. The plates were stained with 0.1% crystal violet solution. Excess stain was washed off thoroughly with 95 % ethanol and plates were kept for drying. Optical density (at 570 nm) was determined using ELISA plate reader. Recorded OD values were considered as an index of attachment to surface thus forming biofilms. The experiment was performed in triplicates and mean OD value was considered. Biofilm formation was determined by formula given below. OD value greater than 0.1 is considered positive for biofilm formation. (Kadurugamuwa *et al.*, 2003).

*BF=AB – CW

(*BF=Biofilm formation, AB is the OD at 570 nm of test bacteria and CW is the OD of control).

Effect of physiological factors on biofilm formation

Biofilm forming ability of isolated *Streptococcus sp.* was studied under different physiological parameters like temperature, pH and sucrose concentration.

Effect of temperature on biofilm formation

Overnight grown culture (200 µl) inoculated with 1 ml of TSB medium in microtitre plate and incubated at three different temperatures like 25^0 C, 37^0 C and 55^0 C for 24 hours. Incubated plates were stained with 0.1% crystal violet and optical density (OD) was recorded at 570 nm.

Effect of pH on biofilm formation

Effect of pH was observed using TSB

medium of different pH 4, 7, 10 inoculated with (200 µl) overnight grown culture in microtitre plates and incubated at 37^0 C for 24 hours. After incubation plates were stained with crystal violet and OD was measured at 570 nm.

Effect of sucrose concentration on biofilm formation

Overnight grown cultures (200 µl) were inoculated in TSB media with varying concentration of sucrose like 2%, 5%, 10% and 15% and incubated at 37^0 C for 24 hours. After incubation plates were stained and OD was recorded at 570 nm (Leme, 2006).

Results and Discussion

Isolation of *Streptococcus* from oral flora

Mitis Salivarius (MS) agar was used for isolation of *Streptococcus sp.* After incubation, three random colonies were selected and their colony characteristics were recorded (Table 1). Glucan synthesis was also observed on MS agar plate (Fig 2). Gram staining results indicated that all the three isolates were Gram positive cocci in chains (Fig. 3).

Biochemical characterization

Biochemical characterization was performed for the identification of isolates. All the three isolates were catalase negative and sugar fermentation indicated only acid production. Haemolysis pattern was observed using Blood agar indicated γ haemolysis by isolates I, III and haemolysis by isolate II. Results are recorded in (Table 2). On the basis of Gram staining and biochemical tests, the organism was identified as *Streptococcus sp.*

Microscopic observation of biofilm

Microscopic observation of biofilm was performed using tissue culture plate. Chains of *Streptococcus sp.* associated with adherent biofilm were observed under oil immersion objective (Fig. 4).

Screening of isolates for biofilm formation by Congo red agar method

Formation of black colonies with a dry crystalline consistency was observed on Congo red agar considered to be positive for biofilm formation. Black colonies with a dry crystalline consistency were observed for all the three isolates indicated biofilm formation (Fig. 5).

Biofilm formation assay

Tube assay

Formation of biofilm was observed by tube. *Streptococcus sp.* isolate I and III were indicating strong biofilm formation while *Streptococcus sp.* isolate II indicating moderate biofilm formation concluded on the basis of the attachment of biofilm (Fig 6) (Mathur *et al.,* 2006).

Microtitre plate assay

Microtitre plate assay was performed for all the three isolates of *Streptococcus sp.* Biofilm formation was measured in terms of adherence to microtitre plate. OD was recorded at 570 nm using ELISA reader indicated biofilm formation in all the isolated *Streptococcus sp.* (Fig. 7). OD values were considered as an index of attachment indicated weak, moderate and strong biofilm formation by isolate II, III and II respectively as per the formula, BF=AB – CW.

Table.1 Colony characteristics of the isolates

Isolates	Colony characteristics							
	Size	Shape	Colour	Elevation	Margin	Opacity	Motility	Gram nature
Streptococcus sp isolate I	1 mm	Regular	Creamy white	Convex	Regular	Opaque	Non motile	Gram positive cocci in chains
Streptococcus sp isolate II	2 mm	Regular	Creamy white	Convex	Regular	Opaque	Non motile	Gram positive cocci in chains
Streptococcus sp isolate III	1 mm	Regular	Creamy white	Convex	Regular	Opaque	Non motile	Gram positive cocci in chains

Table.2 Biochemical characterization

Isolates	Biochemical test						
	Catalase	Sugar fermentation					
		Mannitol		Lactose		Ribose	
		Acid	Gas	Acid	Gas	Acid	Gas
Streptococcus sp. isolate I	- ve	+	-	+	-	+	-
Streptococcus sp. isolate II	- ve	+	-	+	-	+	-
Streptococcus sp. isolate III	- ve	+	-	+	-	+	-

Table.3

Organisms	**Biofilm formation**
Streptococcus sp isolate I	+++
Streptococcus sp isolate II	+
Streptococcus sp isolate III	++

Weak biofilm: +, Moderate: ++, Strong: +++

Fig.1 Biofilm formation

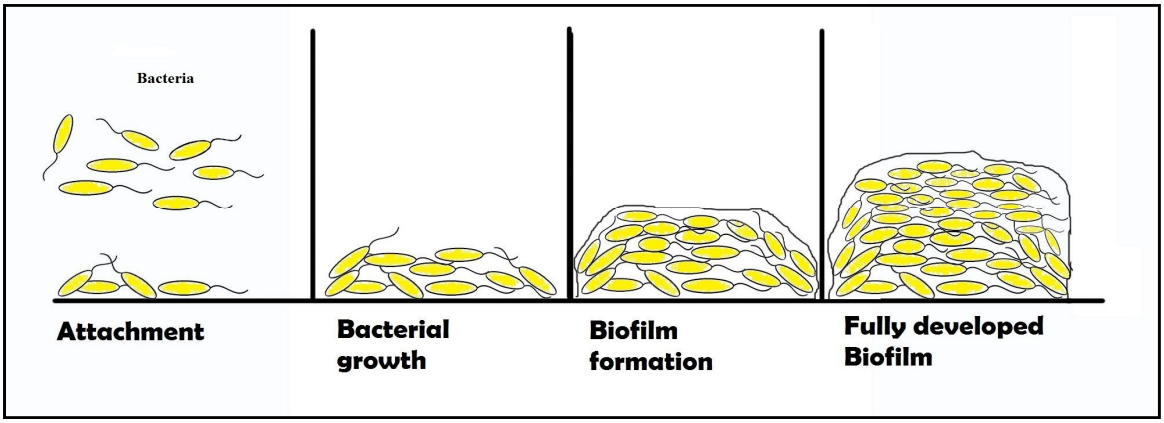


Fig.2 Isolation on MS agar medium

Fig.3 Gram positive cocci in chains

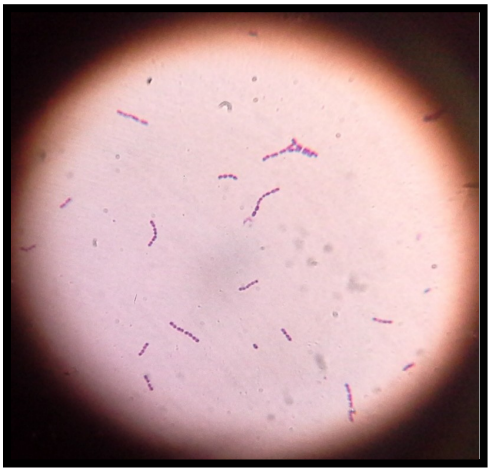


Fig.4 Microscopic observation of biofilm

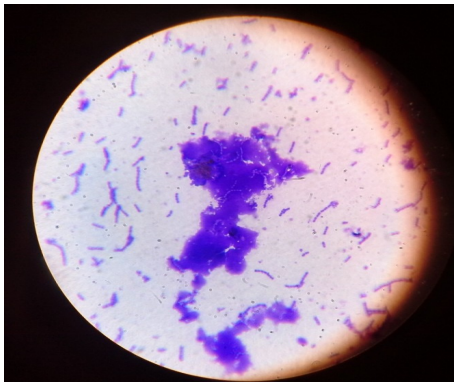


Fig.5 Formation of black colonies on Congo red agar

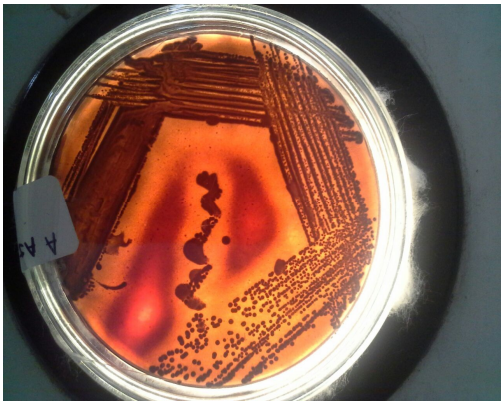


Fig.6 Tube method

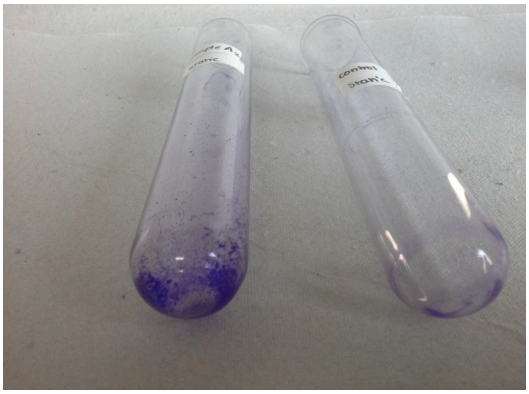


Fig.7 Microtitre plate assay

Fig.8 Effect of temperature on biofilm formation

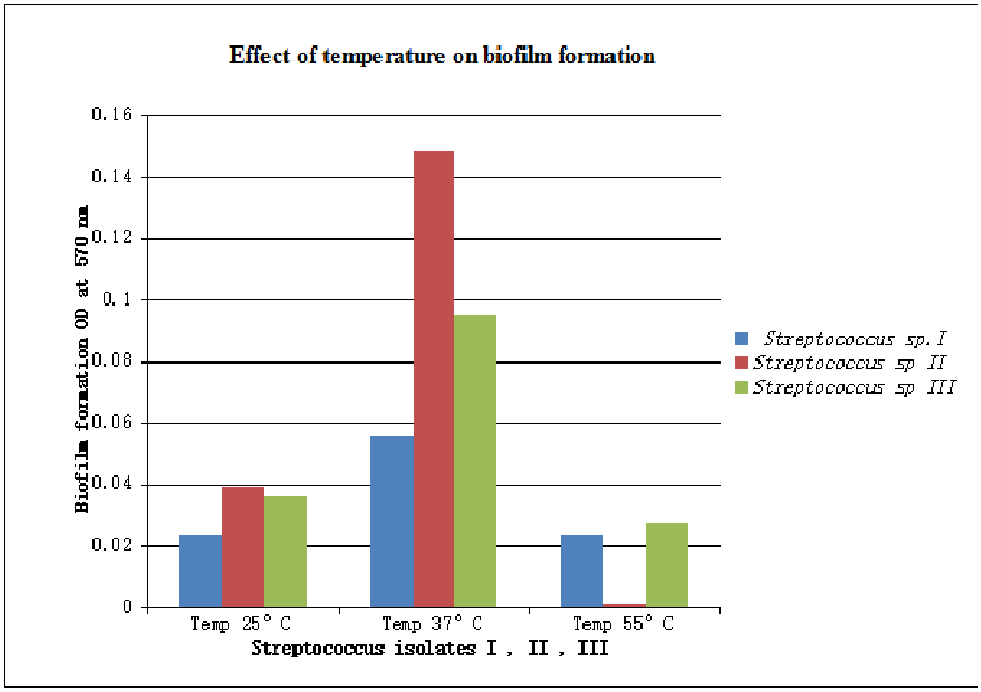


Fig.9 Effect of pH on biofilm formation

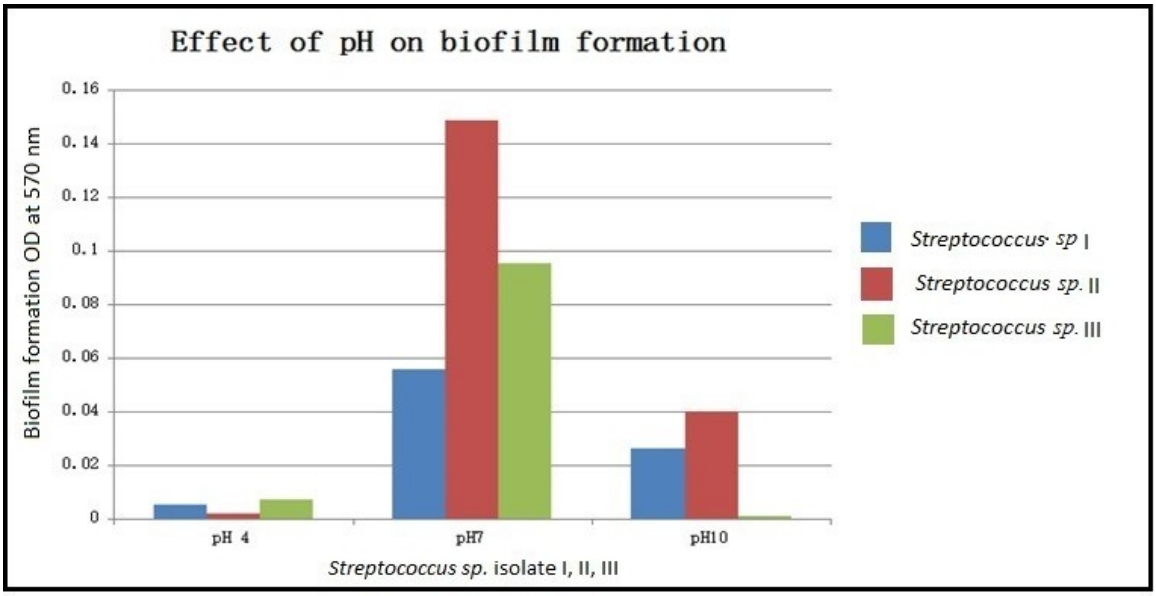
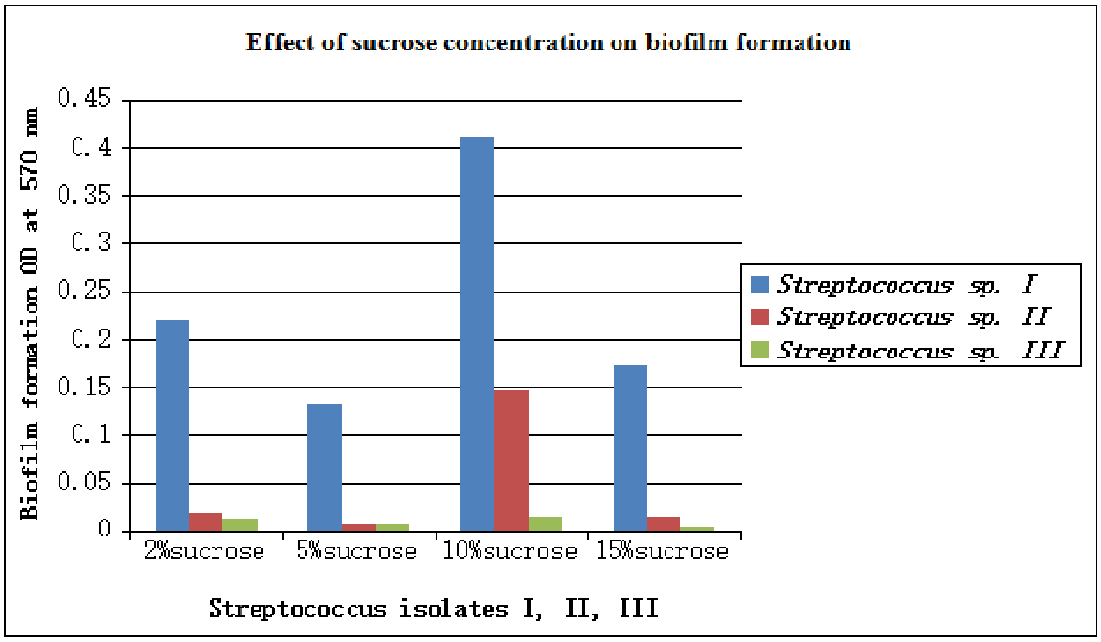


Fig.10 Effect of sucrose concentration on biofilm formation



6 Effect of physiological factors on biofilm formation

Biofilm forming ability of isolated *Streptococcus sp.* was studied under different physiological parameters like temperature, pH and sucrose concentration.

Effect of temperature on biofilm formation

Biofilm formation at different temperature range (25^0 C, 37^0 C, 55^0 C) was studied by measuring absorbance at 570 nm. All the *Streptococcus* isolates shows maximum biofilm formation at 37^0 C, while moderate biofilm formation at 25^0 C and weak biofilm formation at 55^0 C (Fig. 8).

Effect of pH on biofilm formation

Effect of pH on biofilm formation at different pH (pH 4, 7, 10) was studied by measuring absorbance at 570 nm. All the isolates show strong biofilm formation at pH 7, moderate biofilm formation at pH 10 and weak biofilm formation was recorded at pH 4 (Fig. 9).

Effect of sucrose concentration on biofilm formation

Effect of sucrose concentration on biofilm formation was studied at (2%, 5%, 10%, 15%) by measuring absorbance at 570 nm. All the isolates show strong biofilm formation at 10% sucrose concentration while exhibit weak biofilm formation at sucrose concentration 2%, 5% and 15% (Fig. 10).

Microorganisms on wet surfaces have been observed to aggregate and grow into micro colonies from 3-dimensional structures, resulting in a complex biofilm. Therefore aim of present study was to isolate and characterize the biofilm forming *Streptococcus sp* from clinical samples. In this study three isolates from cancer patients was obtained and studied for biofilm

formation. All the three isolates were positive for biofilm formation using Tube assay, micro titre plate assay and Congo red agar method after 24 and 48 hours of incubation at 37^0C. The pH, temperature and sucrose concentration are also known to significantly influence biofilm formation as reported by Czaczyk and Myszka (2007). Our results are in accordance with these reports indicated significant effect of pH, temperature and sucrose concentration on biofilm formation. The present study will help to understand the biofilm formation of oral bacteria and to screen potential antibiofilm agents.

References

Aneja, K.R. 2003. Experiments in microbiology. In: Plant pathology and biotechnology, 4th edn.

Beer, D.D., Stoodley, P. 2006. Microbial biofilms. *Prokaryotes*, 1: 904–937.

Christensen, G.D., Simpson, W.A., Bisno, A.L., Beachey, E.H. 1982. Adherence of slime–producing strains of Staphylococcus epidermidis to smooth surfaces. *Infect Immun.*, 37: 318–26.

Czaczyk, K., Myszka, K. 2007. Biosynthesis of Extracellular Polymeric Substances (EPS) and its role in microbial biofilm formation. *Polish J. Environ. Stud.*, 16(6): 799–806.

Donlan, R.M., Costerton, J.W. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.*, 15: 167–193.

Freeman, J., Falkiner, F.R., Keane, C.T. 1989. New method for detecting slime production by coagulase negative *Staphylococci. J. Clin. Pathol.*, 42: 872–4.

Kadurugamuwa, J.L., Sin, L., Albert, E., Yu, J., Francis, K., DeBoer, M., Rubin, M., Bellinger-Kawahara, C. 2003. Direct continuous method for monitoring biofilm infection in a mouse model. *Infect. Immun.*, 71: 882–890.

Leme, A.F.P., Koo, H., Bellato, C.M., Bedi, G., Cury, J.A. 2006. The role of sucrose in cariogenic dental biofilm formation-new insight. *J. Dent. Res.*, 85(10): 878–887.

Lewis, K. 2001. Riddle of biofilm resistance. *Antimicrob. Agent Chemother.*, 45: 999–1007.

Lewis, K. 2001. Riddle of biofilm resistance. *Antimicrob. Agents Chemother.*, 45: 999–1007.

Marsh, P.D. 2003. Are dental diseases examples of ecological catastrophes. *Microbiol.*, 149: 279–294.

Marsh, P.D. 2004. Dental plaque as a microbial biofilm. *Caries Re.*, 38: 204–211.

Mathur, T., Singhal, S., Khan, S., Upadhyay, U.P., Fatma, F., Rattan, A. 2006. Detection of biofilm formation among the clinical isolates of *Staphylococci*: an evaluation of three different screening methods. *Indian J. Med. Microbiol.*, 24(1): 25–29.

McKenney, D., Hubner, J., Muller, E., Wang, Y., Goldmann, D.A., Pier, G.B. 1998. The ica locus of *Staphylococcus epidermidis* encodes production of the capsular polysaccharide/adhesin. *Infect. Immunol.*, 66, 4711–4720. 27

Patel, R. 2005. Biofilms and antimicrobial resistance. *Clin. Orthop. Relat. Res.*, 437: 41–47.

Patti, J.M., Allen, B.L., McGavin, M.J., Hook, M. 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu. Rev. Microbiol.*, 48: 585–617.