Saudi Journal of Biological Sciences 27 (2020) 2955–2960

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Characterization of biofilm formed by multidrug resistant *Pseudomonas aeruginosa* DC-17 isolated from dental caries

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ARTICLE INFO

Article history:
Received 16 May 2020
Revised 12 July 2020
Accepted 14 July 2020
Available online 18 July 2020

Keywords:
Dental caries
Biofilm
Extracellular polysaccharide
Drug resistant
Antibiotics

ABSTRACT

The present work reports with the screening of biofilm-producing bacteria from the dental caries. The dental pathogens showed resistance against various antibiotics and biofilm forming ability at various levels. Among the bacterial strain, *Pseudomonas aeruginosa* DC-17 showed enhanced biofilm production. Extracellular polymeric substance (EPS) was synthesized by the selected bacterial isolate considerably and contributed as the major component of biofilm. EPS composed of eDNA, proteins and lipids. The total protein content of the EPS was found to be 1.928 mg/mL and was the major component than carbohydrate and DNA. Carbohydrate content was 162.3 mg/L and DNA content of EPS was 4.95 μg/mL. These macromolecules interacted in the matrix to develop dynamic and specific interactions to signalling biofilm to differentiating various environments. Also, the isolated bacteria showed resistant against various commercially available antibiotics. The isolates showed more resistance against penicillin (98%) and were sensitive against amoxicillin. Among the factors, temperature, pH and sugar concentration influenced biofilm formation. Biofilm forming ability of the selected bacterial stain was tested at various pH values and alkaline pH was favoured for biofilm production. Biofilm production was found to be maximum at 40 °C and 8% sucrose enhanced biofilm formation. Biofilm formed by *P. aeruginosa* DC-17 was resistant against various tested antimicrobials and chemicals.

1. Introduction

Dental caries is caused by multiple microbial infections by more than one bacterial species and many bacterial strains were characterized (Paster et al., 2001). An important relationship between the microbes and the host as generally symbiotic in most of the cases (Moore et al., 1985; Geuking et al., 2011). These microbial flora can be free-living or planktonic and mainly organize themselves into a specific consortium of microorganisms referred as biofilm and these microorganisms interact with the use of various extracellular matrix (Monroe, 2007). There are many reasons for the formation of biofilms, these include utilization of cooperative benefits, sequestration to a nutrient-rich area and protective role against harmful conditions (Jefferson, 2004). The important differences of various biofilm producing microorganisms from their planktonic bacteria are their high population, extracellular polysaccharide producing ability, chemical and physical metabolic heterogeneities and surface attachment property (Beer and Stoodley, 2006). Dental caries is the highly common infection caused by certain biofilm producing bacterial strains. These microorganisms interacted with salivary secretions and bacterial strains and carbohydrates from the nutrients (Bowen, 2002; Marsh, 2003). However, very few studies were performed to analyze the bacteria because dental infections and a large number of bacteria involved in dental plaque was not completely characterized (Tanner et al., 1994). Bacteria isolated from the dental caries were characterized by biochemical tests and 16S rDNA sequencing. These methods revealed that the

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2020.07.020
1319-562X/

dental caries bacteria were highly complex (Davey and O'toole, 2000). Streptococcus mutans is one of the well known dental pathogens mainly associated with caries and synthesizes glucan using sucrose (Ito et al., 2020). The synthesis of glucan from sucrose allows the organism to attach strongly to the surface of the tooth and involved in biofilm formation. The gelatinous form of glucan prevents acid diffusion secreted by the bacterial strains colonized in the dental plaque (Kuramitsu, 1993). This mechanism severely damages the hard enamel surface of the healthy tooth. During biofilm formation, interactions between surface and cells critically influenced in colonization of bacterial cells, and the interactions between cells and cells protein mainly influence on biofilm formation and the growth of bacteria (O'Toole et al., 2000).

Oral microbial-plaque microbial consortia are mainly consisted of various different genetic groups of bacterial strains that survive on the surface of the host. These bacterial species specifically communicate using co-adhesion, co-aggregation, metabolic and physiological interactions (Patini et al., 2018; Borkent and Dixon, 2017; Grover et al., 2015). Actinomyces and Streptococci are the important initial colonizing organisms on the surface of the tooth and the interactions between them are very much helpful in biofilm formation. Generally, dental plaque formation happens in a stepwise process, developing as structurally and functionally well organized rich of various microbial communities (Marsh, 2004). Many phases in dental plaque formation including, pellicle formation, various physico-chemical adhering mechanisms between the pellicle and cell surface, receptor mediated attachment and finally co-adhesion resulting to the previously attached microbial cells (Fiorillo, 2020). This highly complex phase on further process extension leads to the formation of biofilm. Also biofilm forming bacteria influenced on chronic bacterial prostatitis treatment and has been reported recently (Bartoletti et al., 2014).

2. Materials and methods

2.1. Collection of sample

Samples were collected from 20 individuals covers various dietary habits, male and female individuals at various age groups. The swabs were taken from gingival, buccal cavity, subgingival and the entire teeth. Then the swab was inoculated Mitis Salivarius broth (g/L, Crystal violet 0.0008; Trypan blue 0.075; Dipotassium phosphate 4; Sucrose 50; Dextrose 1; Casein enzymatic hydrolysate 15, and Peptic digest of animal tissue 5) and nutrient broth medium (Himedia, Mumbai, India).

2.2. Isolation of biofilm secreting microorganisms and morphological characters

The purified bacterial strains were streaked on Nutrient Agar and Mitis Salivarius agar and incubated for 24 h. The bacterial strains were further sub cultured on Nutrient Agar and the morphologically different bacterial strains were characterized.

2.3. Analysis of biofilm forming bacteria

2.3.1. Determination of biofilm formation using Congo red

The selected bacterial strains were cultured on Mueller Hinton Agar medium (Himedia, Mumbai, India) incorporated with Congo red at 0.08% concentration. The plates were incubated for 48 h at 37 °C. The development of black colour colonies indicated the formation of biofilm and non-biofilm producing bacterial isolates developed dark red colonies (Kaiser et al., 2013).

2.3.2. Biofilm bacteria and antibiotic susceptibility

The bacterial strains were cultured in nutrient broth medium and incubated for 18 h at 37 °C. After 18 h, it was placed on Mueller Hinton agar (Himedia, Mumbai, India) plates. The commercial antibiotics such as penicillin, oxacillin, amoxicillin, ciprofloxacin, cotrimoxazole, ampicillin and aztreonam. Antibiotic sensitivity analysis was carried out by using the Kirby-Bauer method based on the guidelines given by Clinical Laboratory Standard Institute (Bauer, 1966).

2.4. Characterization of the bacterium

Identification of bacterial strain was performed using *16S* rDNA gene sequence analysis. Genomic DNA of strain DC-17 which showed maximum biofilm forming ability was extracted using Qiagen DNA extraction kit and amplified using the forward (AGAGTTTGATCMTGGCTCAG, 5′ to 3′) and reverse (ACGGCTACCTTGTTACGA, 5′ to 3) primer. A thermal cycler machine was used to perform Polymerase Chain Reaction and a vial contains 50 μL reaction mixtures consists of 100 μM of each dNTP, 2 mM $MgCl_2$, 2.5 U Taq polymerase, 0.2 μM of forward and reverse primer and 2 μL template DNA. The amplified 16*S* rDNA gene product was amplified, sequenced and the gene sequences were submitted to Genbank database.

2.5. Microscopic analysis of the biofilm

Biofilm formation was determined using a microscope. This experiment was performed using a tissue culture plate. The bacterial strains were grown in tissue culture plate containing Trypticase Soy Broth (TSB) (Himedia, Mumbai, India). The plate was incubated at 37 °C for 24 h. After 24 h incubation of culture plate the plates were drained. The tissue culture plates were completely washed with PBS (phosphate buffered saline, 0.05 M, pH 7.3) and crystal violet was added to stain the plates. The excess strain was dried off with double distilled water and examined using microscopic observation.

2.6. Influence of physiological and nutrient factors on the development of biofilm

The bacterial strain was incubated in microtitre plate containing TSB at various pH values (4–10). The microtitre plate was incubated 37 °C for 24 h. After 24 h, it was stained with crystal violet and washed with double distilled water. The effect of temperature on biofilm formation was analyzed by incubating the selected bacterial strain in TSB medium at various pH ranges (20–45 °C) for 24 h. The absorbance of the sample was analyzed at 570 nm against blank. Influence of sucrose concentration (2%–10%, w/v) was studied by supplementing sucrose at various levels in TSB and the optical density was observed at 570 nm.

2.7. Characterization of EPS isolated from the matrix of biofilm

The selected bacteria were cultured in Nutrient broth medium and the medium was diluted using sterile Nutrient broth medium at 1: 100 ratio. Then 50 mL culture was poured in to an Erlenmeyer flask with glass beads (2.5 mm diameter). The Erlenmeyer flask was incubated at 37 °C for 72 h. After 3 days of fermentation, spent culture medium was decanted and the collected glass beads were carefully washed with buffer (PBS, 0.1 M and pH 7.2) to completely remove planktonic cells. Finally, biofilm formed on the glass beads were carefully vortexed and obtained disintegrated biofilm. The sample was centrifuged at 15,000 g for 20 min for the extraction of EPS. The collected pellets were mixed with 25 mL sulphuric acid in ice cold condition and glass homogenizer was used to break bio-

film. The sample was stored at 2–8 °C for about 4 h with constant stirring and was centrifuged at 10,000 g for 15 min. Total EPS content was analyzed as described by Jiao et al. (2010). Total carbohydrate level of EPS was analyzed and total protein content was tested as suggested by Bradford (1976). DNA was extracted and estimated by standard method.

2.8. Resistance of biofilm of P. aeruginosa to antimicrobials and other chemicals

Antimicrobial resistant and chemicals of biofilm were analyzed. To evaluate antimicrobial resistance, biofilm was established for 72 h were exposed for 10 min to phosphate buffered saline (pH 12, 0.1 M) with sodium hypochlorite (1%), sodium hydroxide (1%), EDTA (40 mM) and chlorhexidine digluconate (2%), respectively. The sample was incubated for 10 min and cell membrane integrity was studied.

3. Results and discussion

Dental caries generally harbours various bacterial strains and represents a highly complex biological system. The bacterial strain has been characteried by *16S* rDNA gene sequence analysis and identified as *Pseudomonas aeruginosa* DC-17. In dental caries various bacteria have been reported. Among various bacterial species, *Bacillus* is generally considered as transient microbial biota in dental samples (Marsh and Martin, 1992). *Pseudomonas aeruginosa* is an opportunistic pathogenic bacterium and is widely investigated for its ability to produce biofilm (Klausen et al., 2003). Many extracellular DNA and exopolysaccharides take part to the structure of the biofilm of bacterial strain, *P. aeruginosa* and has been described previously by Ghafoor et al. (2011). Microorganisms from saliva showed protective roles against integration and colonization of bacteria (He et al., 2011). The growth pattern of biofilm influences on maturation, dispersion and attachment (Sauer et al., 2002). Biofilm-forming bacteria mainly synthesize EPS. The biosynthesized EPS have numerous functions, including, mature biofilm structure, microcolony maintenance, and enhanced resistance to various disinfectants and to environmental stress (Salama et al., 2016; Costa et al., 2018).

3.1. Antibiotic susceptibility of biofilm farming bacteria

In this study, *Streptococcus*, *Staphylococcus* and *Pseudomonas* were detected in large numbers (Fig. 1). These bacterial species have been detected from human infections previously (Archer, 2000). The microorganisms such as, *S. epidermidis,* and *P. aeruginosa* were reported previously (Cheung and Ho, 2001). The bacterial strains from the genus *Pseudomonas* co-aggregated with other *Streptococcus* species (Ciardi et al., 1987). *Pseudomonas aeruginosa* produced EPS and formed biofilm and was resistant against chemotherapy (Costerton et al., 1987; Costerton et al., 1999; Harmsen et al., 2010). The isolated organisms showed resistant against various commercially available drugs. The isolates showed more resistance against penicillin (98%) and were sensitive against amoxicillin other drugs. About 8% bacteria showed resistant against aztreonam and about 27% bacteria showed resistance against ciprofloxacin (Fig. 2). Analysis of antibiotic resistance is useful for the effective clinical treatment. Selection of specific antibiotics is based on the efficacy of the antibiotics for the specific bacterial strains. Dental plaques were pool of bacteria where one by-products acts as nutrient for another group of bacteria (Ready et al., 2003). Some of the bacterial isolates have high percentages of resistant against various antibiotics (Orstavik, 2019). The large number of antibiotic resistant suggests that various bacterial species could specifically involved antibiotic resistance among bacterial flora. The present study revealed that most of the dental strains showed resistance against penicillin. This is because of the presence of basic mechanism, inhibitory barrier to the bacterial cell wall, inability of binding protein to penicillin and production of β-lactamase. Ready et al. (2004) found that about 23% of bacterial isolates from dental caries were resistant against penicillin. The oral bacteria namely, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Prevotella intermedia* were resistant to amoxicillin, azithromycin, clindamycin and amoxicillin (Veloo et al., 2012).

3.2. Characterization of biofilm producing bacterial strains

Twenty three morphologically different bacterial strains were grown on Nutrient agar medium. Nine bacterial strains were mucoid, six strains were smooth and glossy, five strains showed adherent and smooth morphology and three were glossy. Appearance of mucoid, smooth and very glossy colonies on Mitis Salivarius Agar was mainly because of the synthesis of glucan. Among the bacterial strains, 14 bacterial strains were Gram-positive, whereas nine bacterial strains were Gram-negative. All twenty three bacterial strains showed dark black colour colonies on Congo red culture medium. Based on the efficacy of biofilm forming ability one bacterial strain was selected for further studies. The selected bacterial strain was subjected to 16S rDNA sequencing and identified as *Pseudomonas aeruginosa* DC-17. It was isolated from the dental caries. Generally bacteria from the dental caries showed strong resistance to various antimicrobial agents than planktonic mode of life

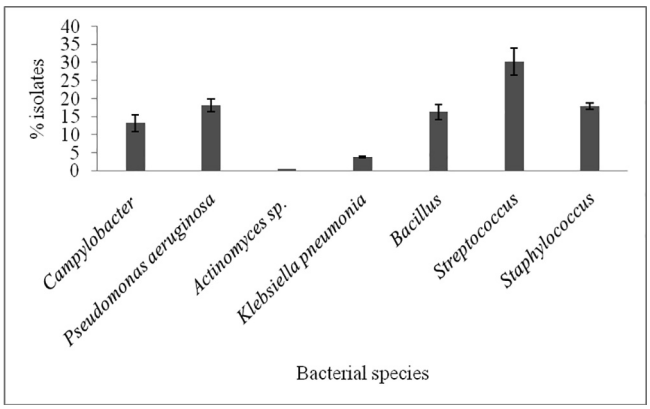

Fig. 1. Bacterial species isolated from dental caries. Error bar: standard deviation.

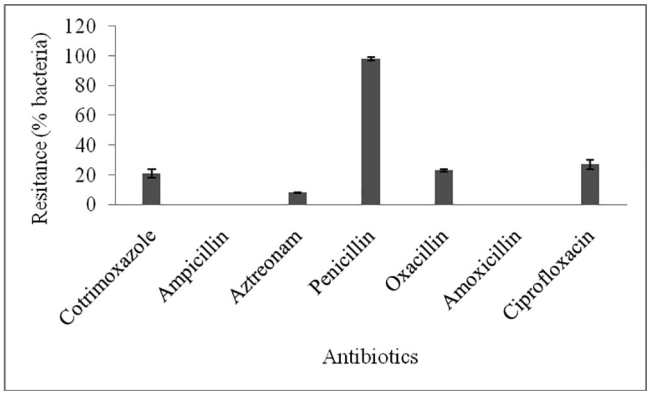

Fig. 2. Antibiotic resistance of bacteria screened from dental caries. Error Bar: standard deviation.

(Stoodley et al., 2002). This was mainly due to the availability of EPS. EPSs effectively inhibits the diffusion of administered antibacterial substances to targeted bacteria, these functioning as an effective protective barrier to plaque forming microorganisms against white blood cells, bacteriophage, surfactant, antibodies and antibiotics (Wang et al., 2018). The bacteria from the group such as, lactobacilli, streptococci and actinomycetes were reported from the dental caries. These microbial consortia were predominant in the small lesions of teeth. The dominant bacterial species in the tooth surface was *Streptococcus* and related to root canal infections. Bacteria such as, *Streptococcus mutans*, *S. mitis*, *S. sobrinus* and *S. sanguis* were isolated from the root caries (Karpiński and Szkaradkiewicz, 2013; Nandakumar and Nasim, 2019). Bacteria from the species and subspecies of *S. sobrinus* and *S. mutans* were generally considered as the mutans streptococci. These mutans can survive in acidic environment, which is an important characteristic feature to survive and contribute cariogenic potential. Actinomycetes such as, *A. viscosus*, *A. naeslundii* can effectively induce root surface caries (Van Houte et al., 1990). Bacteria from the genus *Lactobacillus* such as, *L. oris*, *L. casei* and *L. rhamnosus* were isolated from caries (Samaranayake, 2006). Generally, Lactobacilli are very difficult to grow on tooth surface and mature individually, however, it can be mainly associated with the availability of *Streptococcus mutans* on tooth surface (Lang et al., 2010).

3.3. Production of biofilm at various concentration of sucrose, temperature and pH

Bacterial strain DC-17 was cultured on microtitre plate and biofilm concentration was studied. Biofilm forming ability was maximum after 8 days (Fig. 3). Among the process factors, temperature, pH and sugar concentration influenced on biofilm formation. Biofilm forming ability of the selected bacterial stain was tested at various pH values and alkaline pH was favoured for more biofilm production (Fig. 4). Temperature is also one of the critical factors affecting biofilm production and 40 °C was found to be optimum for the selected bacterial strain (Fig. 5). Sucrose is an important nutrient required for the development of biofilm. Biofilm formation was found to be maximum at 8% sucrose (Fig. 6). Also, EPS matrix supports the microorganisms to utilize various macro and micro nutrients. The quantity of available fermentable sugars in the culture medium determined the composition and structure of EPS. Biochemical test of macromolecular complex revealed the presence of carbohydrate, DNA and protein. The selected bacterial strain analyzed in this study showed varying pattern of biofilm formation. Biofilm production was maximum after 8 days of incubation (1.52 ± 0.052 at 570 nm) and decline later. Temperature is one of the influencing factors affecting EPS production and was maximum at 40 °C (1.743 ± 0.052 at 470 nm). Biofilm formation was found to be maximum in the presence of sucrose at 8% concentration (2.31 ± 0.069 at 470 nm). In a study, Paes-Leme et al. (2006) reported biofilm forming ability of dental pathogens. These dental pathogens formed biofilm in the presence of sugars such as,

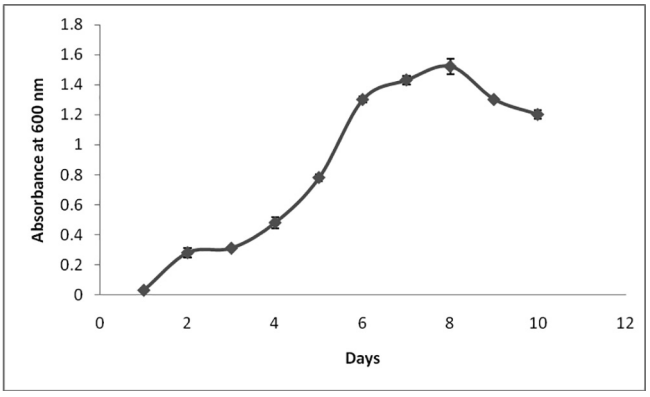

Fig. 3. Biofilm formation of bacterial strain DC-17 on microtitre plate. Error bar-standard deviation.

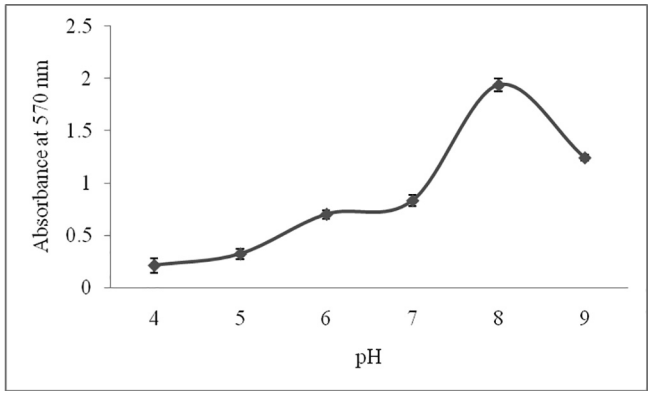

Fig. 4. Effect of pH on biofilm production. The strain DC-17 42 was cultured in Trypticase Soy Broth and the absorbance was read at 570 nm. Error bar: standard deviation.

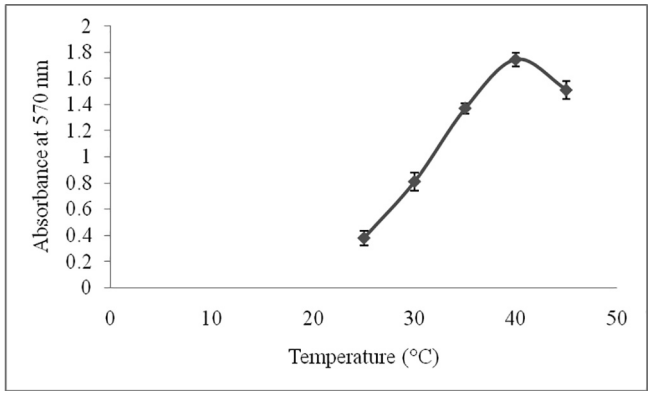

Fig. 5. Effect of temperature on biofilm production. The strain DC-17 was cultured in Trypticase Soy Broth and the absorbance was read at 570 nm. Error bar: standard deviation.

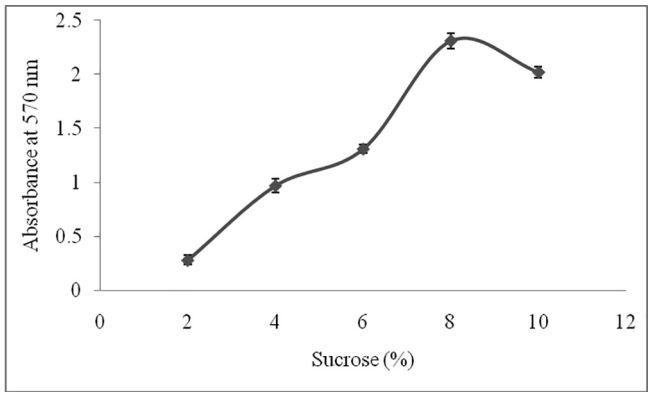

Fig. 6. Effect of various concentrations of sucrose on biofilm production. The strain DC-17 was cultured in Trypticase Soy Broth and the absorbance was read at 570 nm. Error bar: standard deviation.

raffinose and xylose. In some cases, sucrose has been mainly considered as an important sugar and generally acts as a potential substate for the production of intracellular polysaccharide and ESP in dental plaque (Bowen, 2002). Also, the sugars such as, glucose and fructose support the formation of biofilm, however the amount was very low (Leme et al., 2006).

3.4. Isolation of EPS from biofilm and their components

The chemical components of EPS matrix formed by *P. aeruginosa* DC-17 were analyzed. EPS was a mixture of eDNA, proteins and lipids. The total protein content of the EPS was found to be 1.928 mg/mL and was the major component than other macromolecules. Carbohydrate content was 162.3 mg/L and DNA content of EPS was 4.95 μg/mL. In the case of Pseudomonas aeruginosa biofilms, eDNA was reported as an important component of EPS (Alhede et al., 2014). And, few studies also demonstrated the interactions between eDNA and polysaccharide. Hu et al. (2012) reported that eDNA interacted firmly with polysaccharides and enhanced resistance to biological stress and physical strength in *Myxococcus xanthus* biofilms. eDNA has been reported in biofilm of many microorganisms. The bacteria such as, *Enterococcus faecalis*, *Streptococcus mutans*, *Staphylococcus intermedius* and *Pseudomonas aeruginosa* and various species from staphylococci showed eDNA in the matured biofilm. Autolysis of various microbial cells has been reported as an important mechanism of eDNA formation. Qin et al. (2007) reported autolysis of *Staphylococcus epidermidis* and the presence of chromosomal DNA genes from various sites. eDNA in biofilms showed more stability and involved in horizontal gene transfer (Okshevsky and Meyer, 2015). The substances such as amphiphilic compounds, eDNA, adhesion proteins and polysaccharides involved in primary colonization (Nickzad and Deziel, 2014).

3.5. Antimicrobial resistance of biofilm against chemicals

The bacterial strain was grown for 72 h on polystyrene flow-cell chamber and the effect was tested. As described in Fig. 7 exposure of chlorhexidine diclugonate showed potent effect against the bacterial strain. The other chemicals such as, EDTA, sodium hypochlorite and sodium hydroxide also showed significant effect. In endodnontics, EDTA is widely used to remove biofilm layer on teeth (Sen et al., 1995). Also, the recent studies revealed antimicrobial activities of EDTA against biofilm producing microorganisms (Al-Bakri et al., 2009; Venkatesh et al., 2009). The chelating property of EDTA to ions and calcium may critically affect various metabolic pathways in the bacterial cell (Root et al., 1988).

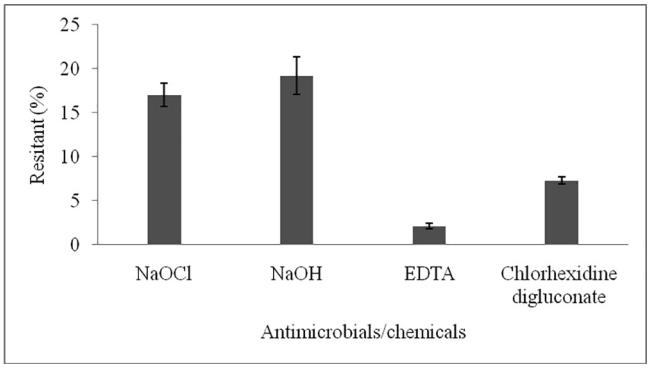

Fig. 7. Antibiotic resistance of biofilm against various chemicals / antimicrobials. Error bar: standard deviation.

4. Conclusion

The present finding revealed that biofilm formation occurs among the bacteria of the dental caries. Although various microbial flora found in the dental caries and these communities showed dynamic resistant against various tested antibiotics. Optimal sucrose level, temperature and pH were useful for the formation of biofilm. Hence, alterations of these three important factors critically affect biofilm formation. Moreover the selected bacterial strain tolerated adverse climatic conditions due to the presence of biofilm and extracellular polysaccharides. Antibiotic sensitivity also varied because of biofilm farming ability of bacteria.

Acknowledgement

The authors thank Bioprocess Engineering Division, Smykon Biotech Pvt LtD for performing the microbial identification and characterization experiment. P. Agastian thank the Loyola College management for the support. RJ thank Holycross College, Nagercoil for the support. The authors would like to thank the Deanship of Scientific Research at King Saud University for funding this work through research group no. RG1439-044.

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