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Biofilm forming ability of bacteria isolated from dental caries: with reference to *Streptococcus* species

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ABSTRACT

Dental caries is formed by the group of microorganisms attached to a tooth surface embedded in a matrix of polymers. Biofilm plays an important role in the development of dental caries, plaque and other periodontal diseases. The present study is aimed to isolate, identify and characterize bacteria from dental caries. The ability of biofilm formation of these isolates was confirmed by Congo red agar method, Tube method and Microtitre plate method. High biofilm-forming bacteria were identified by 16S rRNA sequencing. *Streptococcus mutans*, *Streptococcus anginosus*, *Enterococcus faecalis* and *Rothia dentocariosa* are the predominant biofilm forming bacteria associated with dental caries.

Keywords:

Dental caries, Biofilm, 16S rRNA sequencing

1. Introduction

Dental caries is a predominant dental disease worldwide. According to World Health Organization (WHO) 60–90% of school going children and more than 90% adults suffer from dental cavities leading to the formation of caries. Therefore, dental caries is a major public health problem globally. It is associated with biofilm formed by bacteria which causes destruction of hard tissue due to the production of organic acid by bacterial fermentation of food debris accumulated on tooth surface resulting in tooth decay. About 700 different bacterial species have been identified from the human oral flora [1]. Two major groups of cariogenic bacteria, oral streptococci (group mutans) and lactic acid bacteria (*Lactobacillus spp*) responsible for the formation of dental plaque are also involved in biofilm formation.

Streptococcus, *Actinomycetes*, *Enterococcus* and *Lactobacillus* species are predominant bacteria for deep dental caries lesions [2]. Amongst them, *Streptococcus* species are the most cariogenic pathogens. They produce short-chain acids which soften hard tissues of teeth. It also synthesizes insoluble extracellular polysaccharides due to the sucrose metabolism which increase their adherence to the tooth surface and boosts biofilm formation process [3]. *Enterococcus faecalis* is responsible for the failure of endodontic therapy due to the formation of oral biofilm. *Rothia dentocariosa* is rare Gram-positive bacteria together with other oral cariogenic bacteria are also accountable for causing dental caries. All these microorganisms work together as consortia for causing dental biofilm.

Biofilm formation is a multi-step process in which planktonic bacteria adheres to the pellicle and aggregation into smaller group of bacteria called as microcolonies. In the second step microcolonies secrete extracellular matrix EPS which help to keep biofilms intact. Third step involves the maturation of biofilm, in this maturation; the microcolonies differentiate into different phenotypes. The differentiation can be stimulated by the accumulation of quorum sensing molecule such as N-acyl homoserine lactone (AHL) and Autoinducer peptide (AIP). Mature biofilms contain many porous layers and water channels providing essential nutrients to the microorganisms. Finally, the microorganisms are released from the biofilm by single cell detachment or cluster of cells detaching [4].

During the biofilm formation bacteria communicate with each other and adhere to substratum. Bacteria are embedded in a self-produced matrix of extracellular polymeric substance that exhibit an altered phenotype compared to planktonic cells [5]. The extracellular polymeric substance EPS is complex having cellulose and β -1, 6-linked N- acetylglucosamine. Secondary signal of c-di-GMP regulated their synthesis their concentration depends on the activity of proteins containing a GGDEF/EAL domain. This protein is a common factor in biofilm development [6]. Bacterial biofilm are associated with 80% chronic infections [7]. Microbial biofilm enhances their resistance to antimicrobial agents due to decreased penetration of antibiotics.

Different factors contribute for development of resistance to biofilm forming bacteria. Biofilm is more protective and resistant to action of different antibiotics. Antibiotic resistance increases in biofilm due to glycocalyx matrix, levels of metabolic activity inside the biofilm, genetic adaptation, efflux pumps and outer membrane structure.

In addition, the expression of specific resistance genes in bacteria associated with biofilm also increases the antibiotic resistance [8].

There are different types of methods described in literature to detect the biofilm formation like Microtitre plate method (MPM), Tube method (TM), Congo red agar method (CRA), bioluminescent assay, piezoelectric sensors, and fluorescent microscopic examination. Some ideal methods can be used in routine clinical laboratories.

Tube method (TM) and Congo red agar method (CRA) are preliminary method for the detection of biofilm in microorganisms. One of the standardized method for quantifying biofilm formation is the microtiter plate static biofilm assay. This method is used for quantifying the extent to which a microbe will attach to an abiotic surface [9].

The purpose of this study is to isolate, characterize the oral biofilm-forming bacteria from dental caries and identify strong biofilm forming bacterial isolates using 16S rRNA sequencing technique. The Assessment of biofilm forming bacteria using different methods like tube method (TM), congo red agar method (CRA) and quantified by microtitre plate method.

2. Materials and Methods

2.1 Isolation and characterization of bacteria

Dental caries samples were obtained from 25 volunteer patients with dental caries from Bharati Vidyapeeth Dental College and Hospital, Pune with the approval of the institutional ethical committee. Swab sticks (sterile) were used for collection of the sample. Sample is then immediately transferred to the sterile PBS solution and processed within 2 hours. An aliquot (100µl) of dental caries sample was spread on Mitis-Salivarius Agar (MSA) plate (Himedia, India) with 20% sucrose and Bacitracin (0.2 U/ml) to inhibit the growth of other bacteria, except *Streptococci* [10]. Cultures were incubated in micro-aerophilic condition (candle jar) at 37°C for 48hrs. Random bacterial colonies were selected from the MSB agar plate for further characterization. Bacterial isolates were subjected to Gram staining, Catalase test, Hemolysis test on blood agar and Rapid differentiation test.

2.2 Screening of the isolates for biofilm formation

2.2.1. Congo red agar method

Biofilm formation by the isolates was checked by using congo red agar (CRA) method. Brain heart infusion broth (BHI) supplemented with sucrose (2%) and congo red (0.8gm/lit) was used. Selected colonies were streaked on CRA plates and incubated for 24 to 48 hours at 37°C. Presence of black colored colonies indicates biofilm forming ability while the non-biofilm producing colonies appear red in color [11].

2.2.2 Tube Assay method

Biofilm formation was also confirmed by tube staining method. Overnight grown culture (200µl) inoculated in BHI broth with 2% sucrose incubated at 37°C for 24 hrs. The tubes were decanted and washed with sterile distilled water. Tubes were stained by 0.1% crystal violet and the excess stain was removed by washing 95% ethanol. The visible blue colored ring on the wall of the tube indicates biofilm formation [12].

2.2.3. Microtitre plate assay

An overnight grown culture of bacteria (150µl) was added to 96-well microtitre plates. BHI broth was used as a negative control. The plates were incubated aerobically for 24h at 37°C. The wells were washed with sterile distilled water three times and plates were vigorously shaken to remove non-adherent bacteria. The attached bacteria were fixed by adding methanol (200µl) to each well. Methanol was removed after 15 min and plates were allowed to dry. Microtitre plates were stained with 0.1% crystal violet for 2 min and the excess stain was rinsed off by placing the plate under running tap water. Plates were air dried and glacial acetic acid 33% (v/v) was added to the wells. Optical density (OD) was recorded using micro ELISA reader at a wavelength of 570nm. The experiment was performed in duplicates and the average reading was considered for further analysis [13].

2.2.4. Rapid differentiation of colonies

Isolated colonies were inoculated on MSB agar and incubated at 37 °C for 48 hours. Mixture of mannitol (10%) and 2, 3, 5-triphenyltetrazolium chloride (TTC) (4%) was spread on MSB agar plates. The color change on MSB plates indicates the presence of *Streptococci* [14].

2.2.5. Identification by 16S rRNA sequencing

Seven strong biofilm forming bacterial isolates were identified by 16S rRNA sequencing method. A single colony from overnight grown culture on BHI agar plate was used for colony PCR. DNA

was extracted and purified by using Wizard Genomic DNA Purification Kit. The 16SrRNA gene was amplified using universal bacterial primer 8F forward: 5'AGAGTTTGATCCTGGCTCAG3' and Reverse: 14925'GGTTACCTTGTTACGACTT3'.

Each reaction contains 1µl purified DNA, 0.05µM 8F and 1492R Primer, 25µL Takara's Prime STAR HS (Premix). PCR condition was adjusted to 90⁰C for 5min followed by 30 cycles of 95⁰C for 30 sec, 47⁰ C for 10 sec and 72⁰C for 90 sec with a final extension at 72⁰ C for 10 min.

The purified product was analyzed by 1.0% Agarose gel electrophoresis. Sequences were subjected to homology search using BLAST against NCBI database [15]. Sequences obtained were submitted to GenBank (NCBI) for accession numbers.

2.2.6. Phylogenetic classification

Phylogenetic analysis was performed by using 16S rRNA sequences. All sequences of the bacterial isolates and their homologous sequences were subjected to multiple sequence alignment using MEGA-6 tool. Further, alignment was used for constructing the phylogenetic tree.

3. RESULTS

3.1. Isolation and characterization of bacteria

Isolation of oral bacteria from dental caries was carried out by using Mitis-Salivarius agar (MSA) aimed to isolate *Streptococci*. Randomly selected 24 bacterial isolates grown on MSB agar were used for further characterization. After Gram staining 20 bacterial isolates were found to be Gram-positive, spherical or ovoid cocci arranged in pairs and chains. These were non-sporing, non-motile. Remaining 4 isolates were Gram-negative. All the isolates indicated gamma hemolysis on blood agar. Characteristics of the isolates are given in Table 1.



Fig. 1: Gram Staining



Fig. 2: Colonies on MSBA



Fig. 3: Gamma Hemolysis on Blood agar

3.2. Biofilm forming ability of isolates

3.2.1. Congo red agar method

Using CRA method, dark black colonies with dry crystalline consistency was produced by 14 bacterial isolates indicated high biofilm formation while moderate biofilm was shown by 4 isolates. The weak biofilm was shown by 1 isolate while no biofilm formation was observed in 5 bacterial isolates.

3.2.2. Tube assay method

Biofilm formation is indicated by the visible blue color ring formed on the wall of the tube by this method. Similar results were obtained using Tube assay method where 8 isolates indicated strong biofilm, 10 with moderate Biofilm and 4 isolates have shown weak biofilm while no biofilm formation was observed in 2 isolates.



Fig.4: Colonies on congo red Agar



Fig. 5: Tube assay

Table 1: Characterization of bacteria isolated from dental caries

Sr. No.	Characteristics of isolated bacterial colonies					Biofilm formation assay		
	Bacterial Isolates	Gram Staining	Catalase Test	Hemolysis	Rapid differentiation test	Tube assay method	Congo-red agar method	Microtitre plate assay
1.	DS7	Gram -ve	+	Υ	-	++	+	-
2.	DS8	Gram +ve	+	Υ	+	+	+++	++
3.	DS9A	Gram +ve	-	Υ	+	+++	+++	+++
4.	DS9B	Gram +ve	-	Υ	+	+++	+++	++
5.	DS10	Gram -ve	+	Υ	-	++	+++	+
6.	DS12(A)	Gram +ve	-	Υ	+	+++	+++	+++
7.	DS12(B)	Gram +ve	+	Υ	+	+	+++	++

8.	DS 15(A)	Gram -ve	+	Υ	+	-	-	-
9.	15(B)	Gram +ve	-	Υ	+	+++	+++	+++
10	16B	Gram +ve	+	Υ	+	+++	+++	+++
11	16C	Gram +ve	-	Υ	+	+++	+++	+++
12	17A	Gram -ve	+	Υ	-	++	++	+
13	17B	Gram +ve	-	Υ	-	++	+++	++
14	18A	Gram +ve	-	Υ	+	++	+++	++
15	18B	Gram +ve	+	Υ	-	-	-	-
16	19A	Gram +ve	-	Υ	+	+++	+++	+++
17	20	Gram +ve	+	Υ	-	++	++	+
18	21A	Gram +ve	+	Υ	-	++	+++	++
19	22A	Gram +ve	+	Υ	-	++	++	++
20	22B	Gram +ve	-	Υ	+	++	-	-
21	23A	Gram +ve	-	Υ	-	+	-	-
22	24	Gram +ve	+	Υ	-	++	-	-
23	25A	Gram +ve	-	Υ	+	+++	+++	+++
24	25B	Gram +ve	+	Υ	-	+	++	+

*DS- Dental isolates, Strong biofilm-(+++), Moderate biofilm-(++), Weak biofilm-(+), No biofilm-(-)

3.2.3. Microtitre plate assay

Biofilm formation assay was performed in 96-well microtitre plate assay. Optical density was measured at 570nm. Optical density was measured and value of absorbance is used to correlate the intensity of biofilm formation. Out of 24 isolates, 7 isolates with strong, 7 isolates with moderate and 4 isolates with weak biofilm formation was observed. Results are shown in Fig. 6.

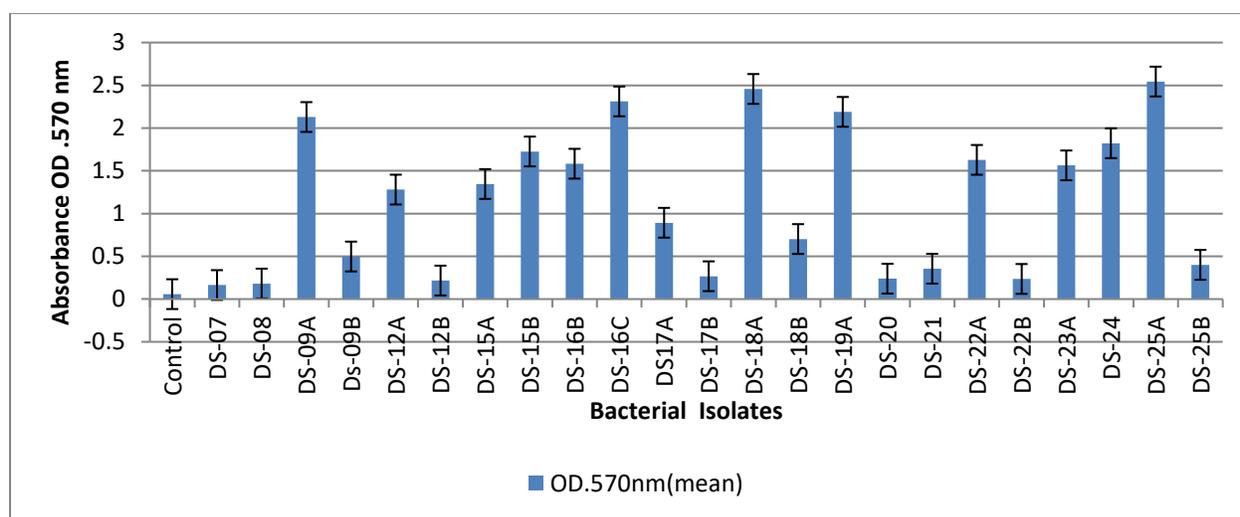


Fig 6: Biofilm formation assay by microtitre plate method

3.2.4. Identification of isolates by 16S rRNA sequencing

Seven strong biofilm forming isolates were identified & sequenced by using 16S rRNA sequencing technique. (Table 2) DNA from bacteria was isolated, amplified by PCR and separated using Agarose gel electrophoresis (1%). (Fig. 7) Purified PCR products were sequenced and searched for homology using BLAST. Two isolates were identified as *Enterococcus faecalis*, two *Streptococcus anginosus*, two *Rothia dentocariosa* and one *Streptococcus mutans*. The results are summarized in Table 2. Results of phylogenetic analysis are given in Fig 8 to Fig 11.



Fig 7: Gel electrophoresis of PCR purified DNA of isolate using 1kb NEB Ladder

Table 2: Identification of bacterial isolates

Sr. No.	Bacterial Isolate	Identified species (based on sequence homology)	Probability	Accession Number
1	DS-25	<i>Streptococcus mutans</i> strain NBRC 13955	100%	MH889143
2	DS-09	<i>Enterococcus faecalis</i> strain ATCC 19433	100%	MH793461
3	DS-12	<i>Enterococcus faecalis</i> strain ATCC 19433	100%	MH793461
4	DS-15	<i>Streptococcus anginosus</i> subsp. <i>whileyi</i> strain CCUG 39159	99%	MH889145
5	DS-16	<i>Streptococcus anginosus</i> subsp. <i>whileyi</i> strain CCUG 39159	100%	MH889145
6	DS-22	<i>Rothia dentocariosa</i> strain ATCC 17931	99%	MH824681
7	DS-24	<i>Rothia dentocariosa</i> strain ATCC 17931	100%	MH824681

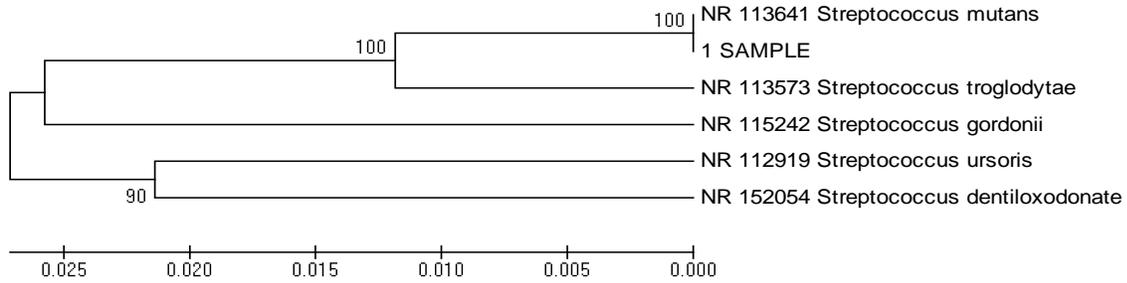


Fig 8: Phylogenetic tree showing homology to *Streptococcus mutans*

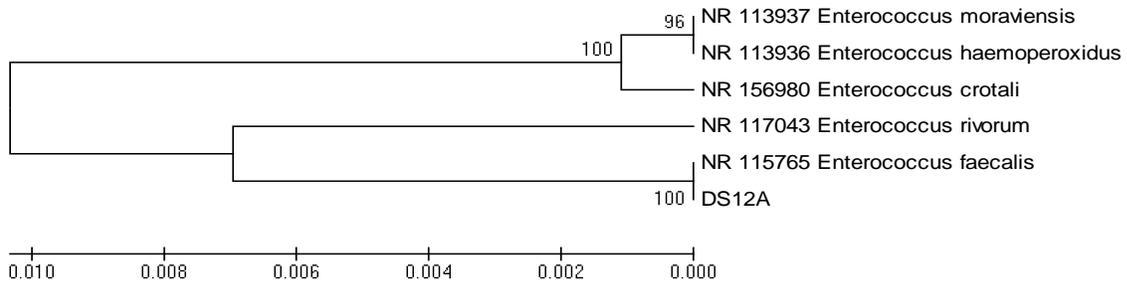


Fig 9: Phylogenetic tree showing homology to *Enterococcus faecalis* strain

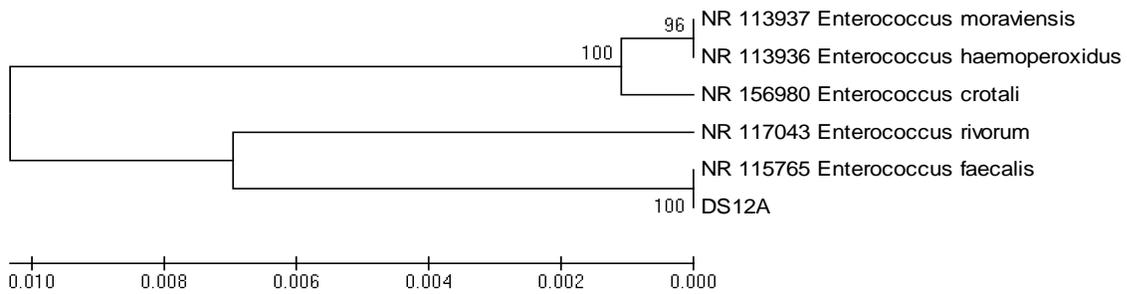


Fig 10 Phylogenetic tree showing homology to *Enterococcus faecalis* strain

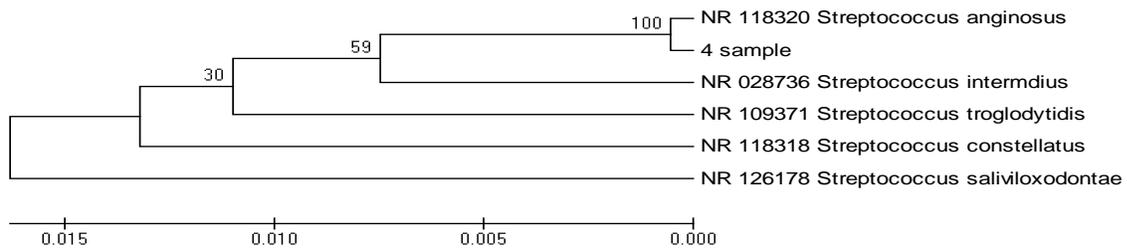


Fig 11: Phylogenetic tree showing homology to *Streptococcus anginosus* strain

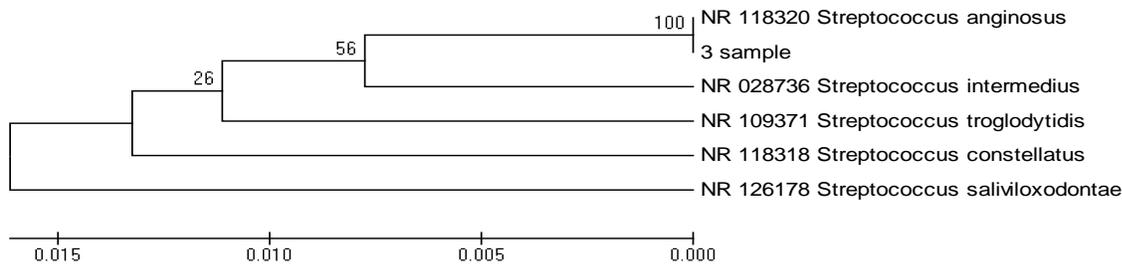


Fig 12: Phylogenetic tree showing homology to *Streptococcus anginosus* strain

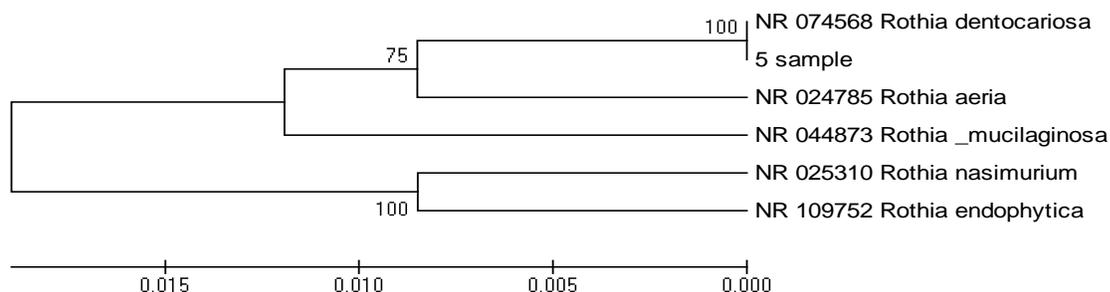


Fig 13: Phylogenetic tree showing homology to *Rothia dentocariosa* strain

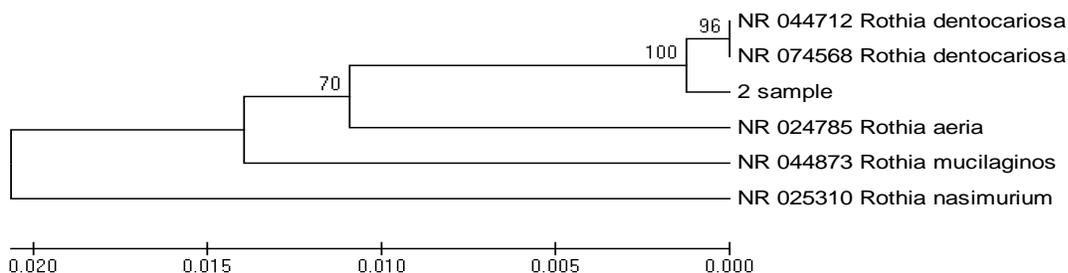


Fig 14: Phylogenetic tree showing homology to *Rothia dentocariosa* strain

4. Discussion

Dental caries is a biofilm-mediated, multi-factorial and chronic infection. The groups of microorganism in caries have diverse types of facultative and obligatory-anaerobic bacteria [9, 10]. Predominant oral bacteria reported are *Streptococci*, *Enterococci* and *Actinomycetes* which are the important causative agent for dental caries [16]. Previous studies have also reported that oral biofilm mostly consists of multiple bacterial strains which correlate our data in the present study. In our studies, biofilm forming bacterial species were identified as *Streptococcus mutans*, *Streptococcus anginosus*, *Rothia dentocariosa* and *Enterococcus faecalis* although the study was intended to isolate only *Streptococcus* species [17].

Earlier reports of biofilm formation studies have shown the highest biofilm formation by *S. mutans* (0.495 ± 0.122). Therefore, *Streptococcus mutans* is considered as etiological agent for the formation of dental caries [18]. as per with these reports strong biofilm forming ability was exhibited by *Streptococcus mutans* by all three methods.

There are some recent reports suggested that apart from *S. mutans* other *Streptococci* such as *Streptococcus oralis*, *Streptococcus anginosus*/*Streptococcus intermedius*, *Enterococcus faecalis* and *Streptococcus mitis* have also shown strong biofilm formation [19]. Similar results were obtained in our study. Along with *Streptococcus mutans*, *Streptococcus anginosus*, *Enterococcus faecalis* and *Rothia dentocariosa* have shown the moderate to strong biofilm formation. Thus, these bacterial isolates are found to be responsible for development of dental caries.

5. Conclusion

Isolation and characterization of biofilm forming bacteria was performed in this study. Seven bacteria have shown strong biofilm formation, eight with moderate biofilm formation, six with weak biofilm formation and no biofilm formation in four isolates by microtitre plate method. Strong biofilm forming bacterial species were identified by 16S rRNA sequencing technique reported one isolate of *Streptococcus mutans*, two isolates of *Streptococcus anginosus*, two isolates of *Rothia dentocariosa* and two isolates of *Enterococcus faecalis*. These are the predominant bacteria associated with dental caries.

Conflicts of interest

No conflict of interest.

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