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Isolation and Characterization of Biofilm Producing Organisms from Tooth Surfaces of Children

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Abstract: Dental diseases are recognized as major public health problems throughout the world. The human mouth is colonized by a variety of microorganisms, including bacteria, fungi, and viruses. These microorganisms colonize all oral surfaces, including the tooth surface, forming consortia referred to as oral biofilms. Bacteria in the biofilm produce acid in the presence of sugars which in turn decay the tooth. Early childhood may be the most important time for the future dental health. During this period the primary teeth erupt, bacteria colonize on the teeth and the dental health behaviour begins to form. Dental plaque samples were collected from children of age group 2-12 yrs, using sterile toothpick in sterile transport medium and isolated on Nutrient agar supplemented with 5% sucrose and Brain heart infusion agar. All the dental plaque pathogens were analysed for their biofilm formation qualitatively and quantitatively. The prominent biofilm producers were identified on the basis of their morphological, cultural and biochemical characteristics. The biofilm producers isolated from dental plaque were identified as S.mutans and Bacillus spp.

Keywords: S.mutans, Biofilm, Dental diseases, Plaque.

I. INTRODUCTION

Dental caries represents one of the most prevalent and costly biofilm-dependent diseases that afflict children and adults worldwide. The disease, manifested clinically as cavities, is a prime example of the consequences arising from interactions on (tooth) surfaces between microorganisms, microbial products, host (saliva) and diet (sugar), leading to the establishment of pathogenic biofilms, or dental plaque, that causes tooth decay (Hyun K. et al., 2014). Plaque-related diseases are probably the most common bacterial diseases occurring in man.

Dental caries (dental decay) is a destructive condition of the dental hard tissues that, if unchecked, can progress to inflammation and death of vital pulp tissue, with eventual spread of infection to the periapical area of the tooth and beyond. The disease process involves acidogenic plaque bacteria, including Streptococcus mutans, Streptococcus sobrinus and Lactobacillus spp., whereas periodontal diseases can involve both the soft and hard tissues and are the most common inflammatory destructive conditions that affect man.

They are initiated by components of the plaque that develops on the hard root surface adjacent to the soft tissues of the supporting periodontium and may be confined to the gingiva (gingivitis) or extend to the deeper supporting structures with destruction of the periodontal ligament and the alveolar bone that supports the teeth (Robert A. et al., 2009).

Several studies have assessed the prevalence of early childhood caries (ECC) throughout the world, in a comprehensive review, observed a prevalence rate from 1 to 12% in developed countries and prevalence as high as 70% in developing countries or within disadvantaged populations. ECC should be considered a multifactorial disease whose etiology involves biological, psychosocial and behavioural factors (Ana P. et al., 2007).

Oral biofilms mostly consists of multiple bacterial strains include mainly the Streptococcus species i.e. Streptococcus mutans which has major role in the formation of dental caries. In presence of sugar these bacteria ferment the dietary sugars producing acid and lowering the pH of the mouth, leading to demineralization of the teeth which is the initial step in oral biofilm formation. Whereas the mouth acidity is maintained by base producing bacteria like Streptococcus Sangius and Streptococcus oralis which neutralize the acid produced maintaining the oral health. If the balance between these two acid and base producing organisms is disturbed the oral health is hampered (Kaustubh S. et al., 2015).

The present study aims at the isolation and identification of biofilm forming organisms from the tooth surfaces of children which are generally proned to dental caries.

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II. MATERIALS AND METHODS

A. *Sample Collection And Isolation Of Dental Plaque Pathogens*

Total 44 dental plaque samples were collected from children of age 2-12 years with due consent from their parents. The consent form contained various criteria including brushing habits, eating habits, use of (Toothpaste/Toothpowder/Mouthwash) along with age and sample collection date and time. Since eating habits and brushing habits play important role in dental caries so both were recorded. The plaque samples were collected with the help of sterile toothpick after 2-3 hrs of brushing in Sterile Ringers solution. The collected samples were immediately carried to the laboratory and isolated on sterile Nutrient agar with 5% sucrose and also on Brain Heart Infusion agar in anaerobic chambers.

B. *Detection Of Biofilm Formation Ability Of The Isolate*

1) *Congo Red Agar Method (Qualitative):* This method is based on the characteristic cultural morphology of biofilm-forming bacteria on Congo red medium. The isolates were streaked on the Muller Hinton agar (HIMEDIA) supplemented with 0.8g/l of Congo red dye and incubated for 48 hours at 37°C. The production of black colonies with a dry crystalline consistency indicated biofilm formation and non-biofilm producing strains develop red colonies (Kaustubh S. et al., 2015).
2) *Modified Tube Assay Method (Quantitative):* Biofilm formation was determined by the tube staining assay (Christensen et al., 1982). Isolates were inoculated in Trypticase soy broth (TSB) with 5% sucrose and incubated for 24 hours at 37°C. The tubes were decanted and washed with phosphate buffer saline (PBS) (pH 7.3), dried and Stained with 0.1% crystal violet. Excess stain was removed by washing the tubes with deionized water. Formation of biofilm was confirmed with the presence of visible film on the wall and bottom of the tube. The biofilm which was formed was extracted using alcohol and was then quantified using colorimeter at 570 nm.
3) *Identification of Biofilm Producers:* Isolates were characterized morphologically by Gram staining. The isolates were preceded for cultural and biochemical characterization as per Bergey's Manual of Determinative Bacteriology.

III. RESULTS AND DISCUSSIONS

A. *Isolation Of Dental Plaque Pathogens*

Total 113 isolates were obtained after the isolation of the dental plaque samples on Nutrient agar with 5% sucrose and Brain heart infusion agar (Fig. 1a and 1b).Sucrose causes major biochemical and physiological changes during the process of biofilm formation, which, in turn, enhance its caries-inducing properties. Sucrose promotes an increase in the proportions of mutans streptococci and lactobacilli and, simultaneously, a decrease in S. sanguinis levels as a result of the pH fall caused during the fermentation of this carbohydrate. Acid production from sucrose metabolism disrupts the balance of the microbial community, favoring the growth of cariogenic species (Paes L. et al., 2006).Dental caries is a diet dependent bacterial disease and the properties of the biofilm formed on tooth surfaces may explain the different caries patterns found. Among the dietary components, sucrose (sugar) is considered the most critical, regarding the biochemical and microbiological change that it induces in dental plaque composition (Nobre M. et al., 2005). The early colonizers of the tooth surface include members of the genera Streptococcus, Actinomyces, Haemophilus, Neisseria, and Veillonella. These bacteria adhere to the acquired enamel pellicle by specific and non-specific molecular interactions between adhesins on the cell and receptors on the surface. Once established, the microflora at a site remains relatively stable over time despite regular minor perturbations to the oral environment. This stability (termed "microbial homeostasis") stems not from any metabolic indifference among the components of the microflora, but rather results from a dynamic balance of microbial interactions, including both synergism and antagonism (Marsh P., 1994).

B. *Detection Of Biofilm Producing Dental Plaque Pathogens*

Congo red agar is nothing but Mueller Hinton agar supplemented with Congo red dye. The method is used for the detection of biofilm production where the biofilm formers produce black crystalline dry colonies and non-biofilm producers produce colourless colonies. Out of 113 isolates, 27 showed black crystalline colonies with a dry crystalline consistency which indicated biofilm formation ability (Fig.2). Similar method has been used by Kaustubh S. et al., (2015) who carried out isolation and characterization of oral biofilm forming microorganisms from healthy individuals and confirmed the biofilm formation by Congo red agar.

All the isolates which showed positive qualitative test for biofilm formation were studied for their gram nature and catalase test. Quantitative screening of biofilm producers was done by using modified tube assay method. The tube assay is generally performed in qualitative tests but here the test was converted into quantitative test. The biofilm which was adhered to the test tube was extracted and quantified in the terms of absorption (Fig.3). Out of 27 isolates, 19 were Gram positive cocci and 8 were Gram

positive rods. Only 2 Gram positive cocci did not show catalase activity while all the remaining organisms showed catalase activity. Catalase test was also performed on which the 5 biofilm producers were selected. Oral microbiota consists of mostly anaerobes or facultative anaerobes, so such organisms are generally catalase negative.

The biofilm formation was evaluated quantitatively by tube assay. The tubes were stained with crystal violet and extracted with alcohol for absorption study. Biofilm production by gram positive rods is shown in Fig.4. Among gram positive catalase negative cocci i.e. isolate 65 and 97 showed highest absorbance of 1.34 and 1.38 units respectively (Fig.5). On the basis of biofilm production, 5 isolates were selected, out of which 3 were catalase negative and 2 were catalase positive. Similar work has been done by Kirti J. et al., (2013) who carried out characterization of two biofilm-forming bacteria isolated from the oral cavity. Kaustubh S.et al., (2015) has carried out isolation and characterization of oral biofilm forming microorganisms from healthy individuals and evaluated biofilm formation by tube assay. The adsorption of bacteria to solid surfaces has been studied by marine and soil microbiologists and subsequent investigators noted that the initial phase of attachment of marine bacteria to glass surfaces involved a loose association. This was followed by a phase in which cells became more firmly attached and this process was often associated with the synthesis of mucilagenous or holdfast material (Gibbons R. et al., 1980). The production of extracellular glucosyltransferase by Streptococcus mutans and Streptococcus salivarius has been reported to be important to the binding of these streptococci to dental surfaces. These extracellular enzymes also transfer adherent growth to normally nonadherent bacteria by the adsorption of the extracellular glucosyltransferase onto the surface of the cell, with subsequent binding to the dental surface (Christensen G. et al., 1982).

C. Identification of Biofilm producers

In the present study the selected 5 dental plaque pathogens were identified on the basis of morphological, cultural and biochemical tests. Out of 5 isolates, 2 were gram positive thick rods and 3 were gram positive cocci in chains. All the isolated gram positive rods were non acid-fast and catalase positive. 2 gram positive rods showed spore formation ability and starch hydrolysis. Spore formation and biochemical characteristics all the isolated gram positive rods coincided with the genus Bacillus and hence confirmed as Bacillus. Isolate 54 was labelled as Bacillus spp. 1 and isolate 75 as Bacillus spp. 2. Amongst 15 gram positive cocci 3 were catalase negative were further characterized on the basis of biochemical tests. Negative catalase test indicates presence of Streptococcus species. Result of the biochemical test of selected gram positive cocci is shown in Table 1. As per Bergey's Manual of Determinative Bacteriology w.r.t. morphological, cultural and biochemical tests, the isolates were identified as Streptococcus spp. Hemolysis tests were also performed to check their hemolysis pattern and S.mutans showed gamma pattern of hemolysis. Kirti J. et al., (2013) performed the work which deals with the studies on characterization of two biofilm-forming bacteria isolated from the oral cavity. The organisms isolated were identified as Pseudomonas aeruginosa and Bacillus subtilis. Kaustubh S. et al., (2015) carried out characterization of oral biofilm forming microorganisms from healthy individuals and identified on the basis of catalase, IMViC, sugar fermentation as Streptococcus spp., Staphylococcus spp., Enterobacter spp., and Pseudomonas spp.

Thus, the present study concludes the prevalence and association of biobilm forming pathogens with dental caries in children which affects their oral health.

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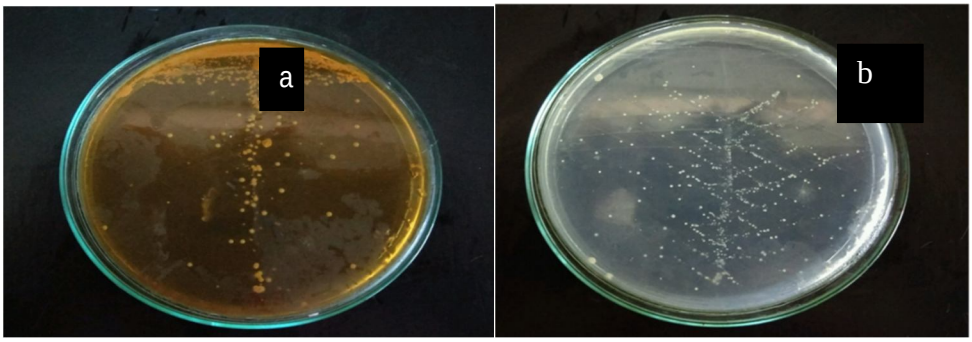


Fig. 1: Isolation of dental plaque pathogens on a) Brain heart infusion agar b) Nutrient agar with 5% sucrose.

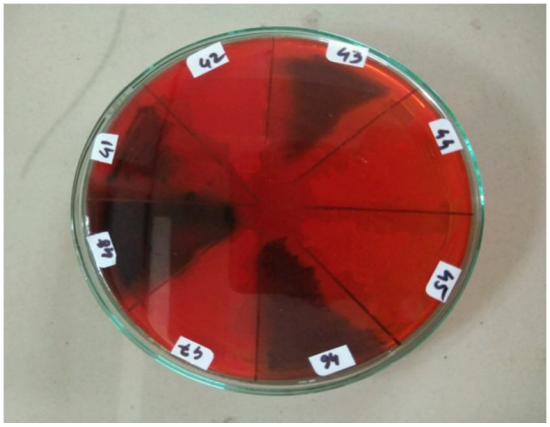


Fig. 2: Qualitative detection of biofilm producing dental plaque pathogens using CRA plate.

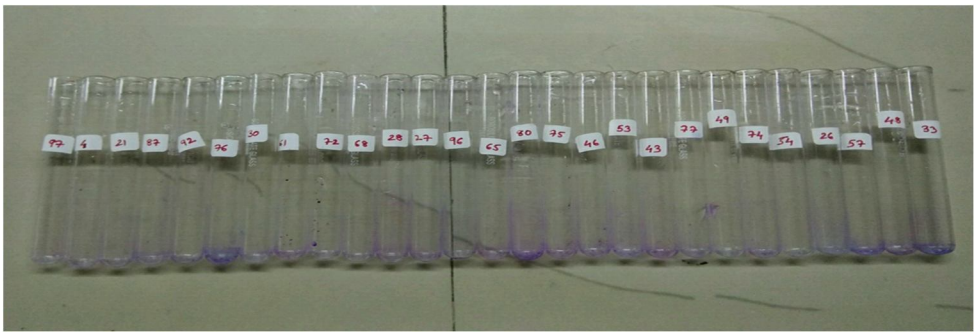


Fig. 3: Determination of biofilm formation by modified tube assay method.

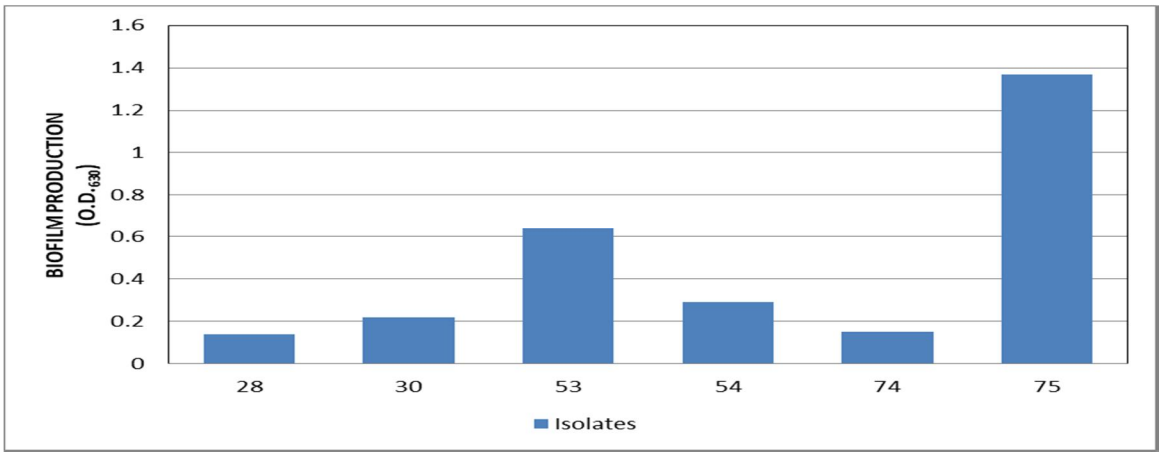


Fig.4: Biofilm production by gram positive rods isolated from dental plaques.

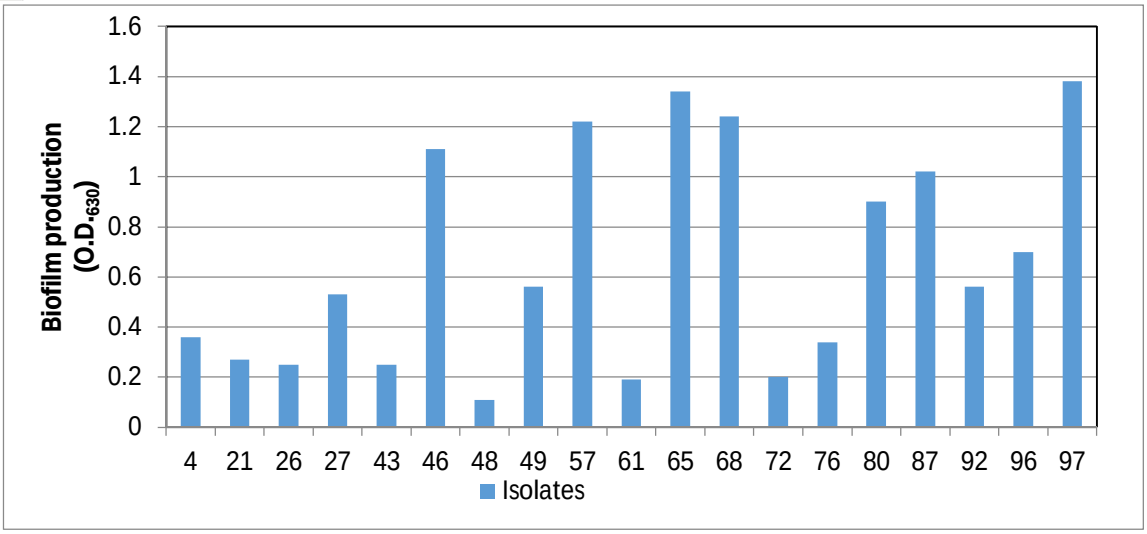
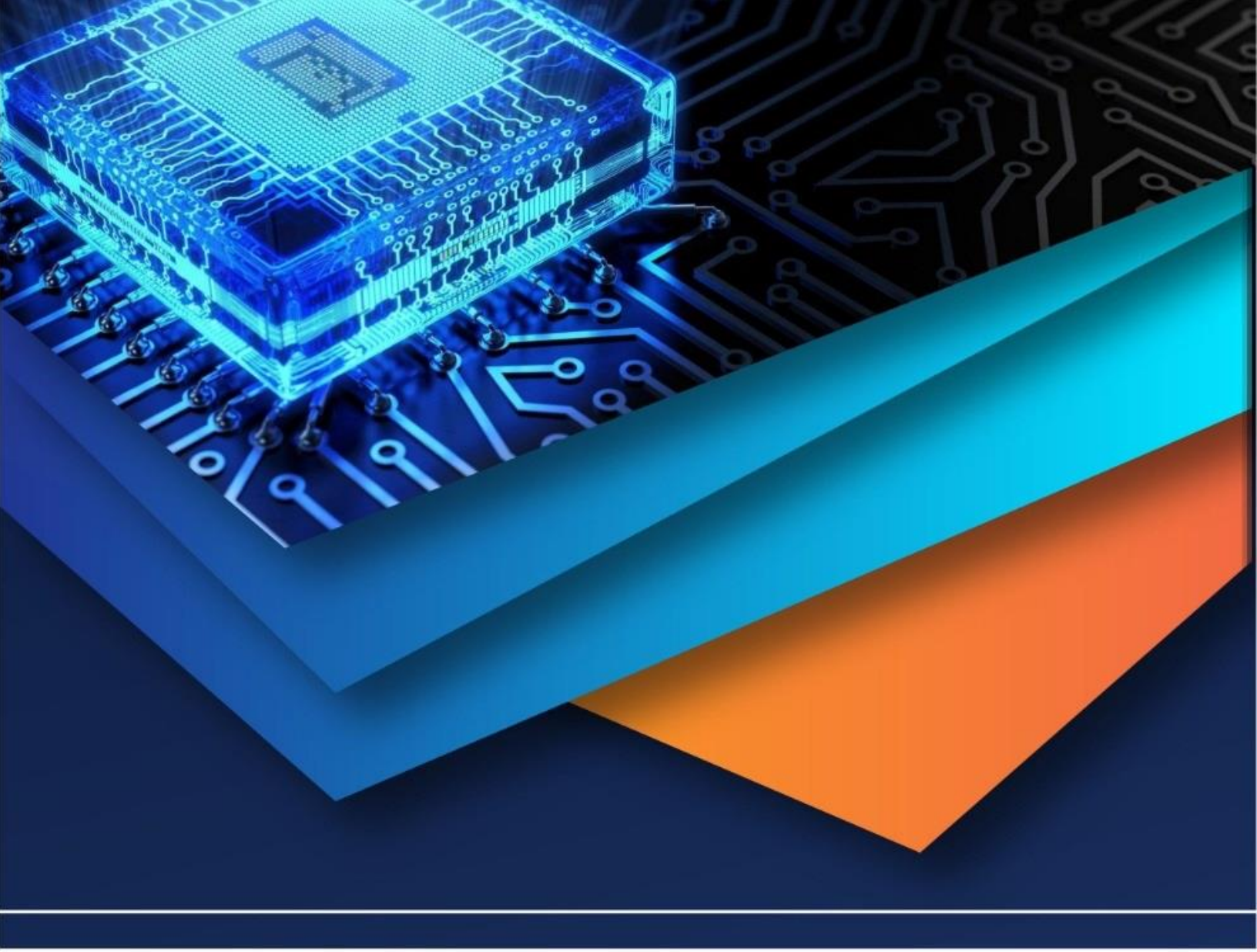


Fig.5: Biofilm production by gram positive cocci isolated from dental plaques.

Table 1: Biochemical characteristics of biofilm forming Gram positive cocci isolated from dental plaques.

Tests	Isolates	Isolate 43	Isolate 65	Isolate 97
GROWTH	Aerobic	+	+	+
	Anaerobic	+	+	+
	10^0C	+	+	+
	45^0C	-	-	-
	pH 9.5	-	-	-
	6.5% NaCl	-	-	-
	0.4% Bile	-	-	-
	0.25% Optochin	+	+	+
HEMOLYSIS	Alpha	-	-	-
	Beta	-	-	-
	Gamma	+	+	+
SUGAR UTILIZATION	Inulin	+	+	+
	Mannitol	+	+	+
	Raffinose	+	+	+
	Sorbitol	+	+	+
	Salicin	+	+	+
	Lactose	+	+	+
	Ribose	+	+	+
BILE ESCULIN TEST		+	+	+
VOGUES PROSKEUR TEST		+	+	+
CATALASE TEST		-	-	-



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