ISOLATION, CHARACTERIZATION AND STRESS RESPONSE OF DENTAL PLAQUE FORMING BACTERIA

PROJECT SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIEMENT OF MASTER OF SCIENCE IN LIFE SCIENCE

By

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ABSTRACT

The current work deals with the studies of isolation, characterization and stress response of dental plaque forming bacteria. The most important constituent of biofilm other than bacterial cells is the Extracellular Polymeric Slime matrix (EPS) which is secreted by the bacterial cells themselves. This EPS matrix determines the physical properties of biofilms like attachment, mechanical strength, and antibiotic resistance. In this study, attempts were made to study the stress response of bacteria isolated and their chemotactic response. Efforts were made to characterize the EPS matrix by chemical as well as spectroscopic studies. For stress response studies, the temperature range considered didn't show much variation whereas the biofilm formation reduced with alkaline pH. By biochemical identification methods, the bacterial strains isolated were characterized to be *Staphylococcus aureus*, *Staphylococcus salivarius*, *Streptococcus mitis*, *Streptococcus oralis*, *Bacillus subtilis*. It was also observed that cell attachment was maximum when glucose was used as the sole carbon source. Test for biofilm formation in presence of metal salts of Iron and Zinc showed moderate to high inhibition of film formation. The chemotaxis studies carried out in present work indicates the poor response of two strains towards fructose and sucrose. The EPS characterization results showed that the EPS matrix was made of different biomolecules such as carbohydrate, protein, lipids and nucleic acids. The formation of biofilm was confirmed by the SEM micrographs taken.

I.INTRODUCTION

No bacterium in the living world is an island in itself, they depend on other microbes for energy, carbon and other nutrients (Prakash et al., 2003).Thus throughout the microbial world, bacteria predominantly remain in self produced polymeric matrix enclosed, structured multicellular communities or microecosystems called biofilms, adherent to an inert or living surface, filled with hundreds of other microorganisms opposed to free living planktonic cells. In natural ecosystem more than 99% of microorganisms live in complex biofilms (Costerton et al., 1999).This is a protected mode of growth that allows survival in hostile environment. Biofilms can be defined as a conglomeration of matrix enclosed bacterial cells irreversibly attached to a surface and allowing growth and survival in sessile environment. Commonly visualized as a slimy layer, a number of unique features distinguish them from their planktonic counterparts, to indicate a few, association with living or inert surface, high population densities, highly complex extracellular polymeric matrix, physical, chemical and metabolic heterogeneities etc. Biofilms thus form a primary habitat for the microorganisms. Biofilm can form on any surface, living or inert, e.g. on rocks in water bodies, body surface of aquatic animals, inside water pipes and on medical implants. This survival strategy of the microbes is mediated by mechanical, biochemical and genetic factors (Beer and Stoodely, 2006). These elicit specific mechanisms for initial attachment to a surface, formation of microcolony leading to development of three-dimensional structure of mature biofilm. By virtue of up regulation and down regulation of approximately 40 per cent of their genes, they alter their growth rate, resistance to biocides, antibiotics and antibodies (Costerton et al., 2000). The different form of biofilm viz slimes flocs and microbial mats cause most of the microbial conversions in natural environment. This is one of the major pathogenic strategies of most of the disease causing microorganisms since it renders them resistant to antibiotics and biocides as well as to body's natural immune system. They hinder drug administration and are responsible for chronic bacterial infection, infection on medical devices food and water quality detoriation etc. Formation of a mature biofilm is a multistep process and follows a sequence of events starting from a planktonic mode of existence. It is known that at lower concentrations bacterial cells exist as free living planktonic organisms depending on the surrounding environment for growth and

multiplication (Characlicks, 1988). However, beyond a threshold concentration, these free living planktonic forms start aggregating to form a highly complex three dimensional organization.

The transition from a planktonic to colonial mode of existence involves a pronounced shift in expression of different sets of gene and in some cases expression of genes which remain unexpressed in planktonic forms (Charalicks, 1990). There is a dramatic change in behaviour of cells in their microcolonies. The growth and activity of bacterial cells is significantly enhanced on attachment to appropriate and the phenomenon is called bottle effect. High level organization of bacterial microcolonies requires intracellular signaling or cell to cell communication which can be considered as a morphogenetic mechanism. This phenomenon is called "Quorum Sensing". This phenomenon other than biofilm formation also modulates key biological events e.g. bioluminescence virulence factors, production of toxins and antibiotics etc. In this system of communication bacterial cells detect their population density through autoinducers synthesized and secreted by bacteria. Autoinducers (AIs) trigger the expression of particular genes when population density reaches a critical value (Davies et al., 1998). Commonly encountered autoinducers are N-acyl-homoserine lactones (Greenberg, 1997).

The sequence of events involved in switching of planktonic mode of bacterial existence to colonial form involves formation of a conditioning layer, bacterial adhesion, bacterial growth, biofilm expansion and development into a mature biofilm and detachment of cells into the surrounding medium (Fig 1.) The physiological features of bacteria helpful in biofilm formation include flagella, fimbrie and pilli which bridge between the bacteria and the conditioning film. The major constituent of biofilm other than bacterial cells is the Extracellular Polymer Slime matrix or the EPS which is a highly hydrated matrix produced by the cells themselves within which the cells remain embedded. While the physiological properties of cells are determined by the cells, the physical properties are largely determined by the EPS. EPS is now known to be composed of proteins, polyuronic acids, nucleic acids and lipids other than polysaccharides which form the major bulk of the matrix (Decho, 1990; Lopez, 1993; Schmidt and Ahring, 1994). Properties of biofilms viz; attachment, detachment,

mechanical strength, antibiotic resistance and exo-enzymatic degradation activity can all be attributed to the EPS matrix.

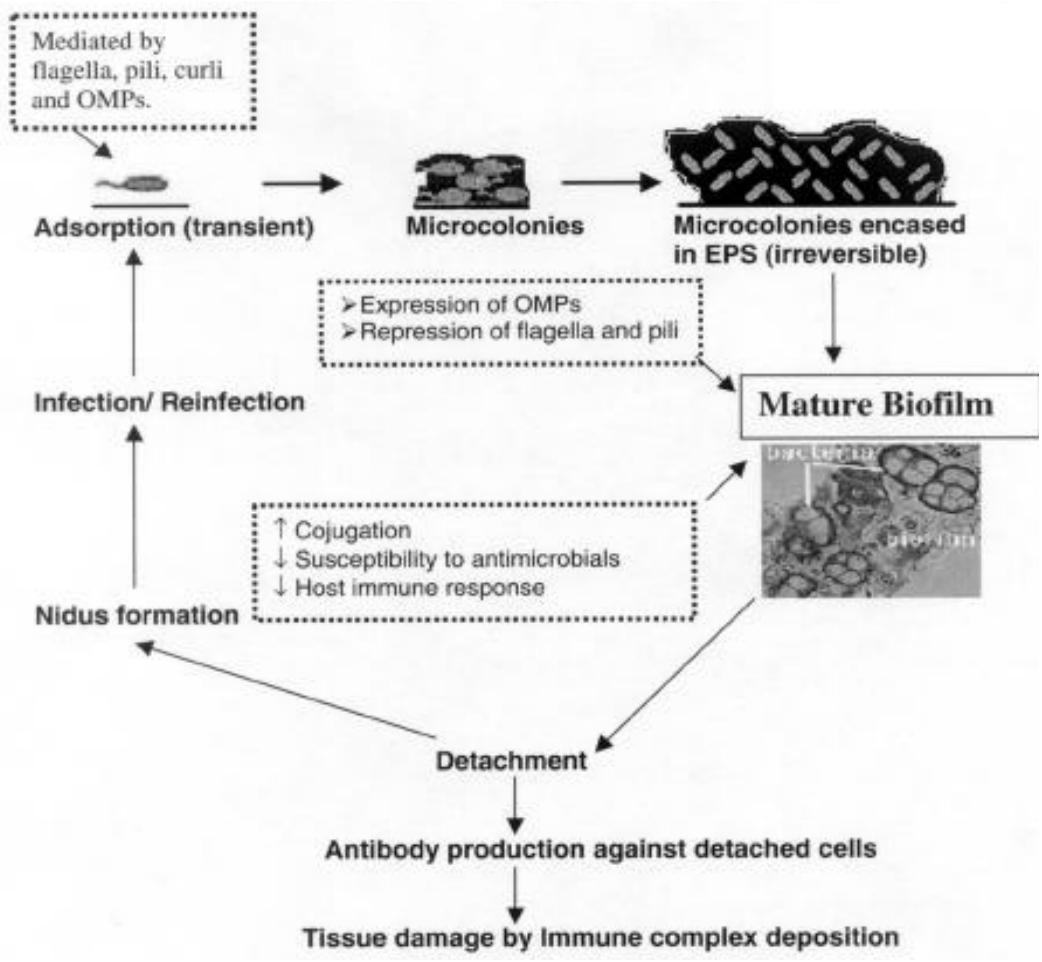


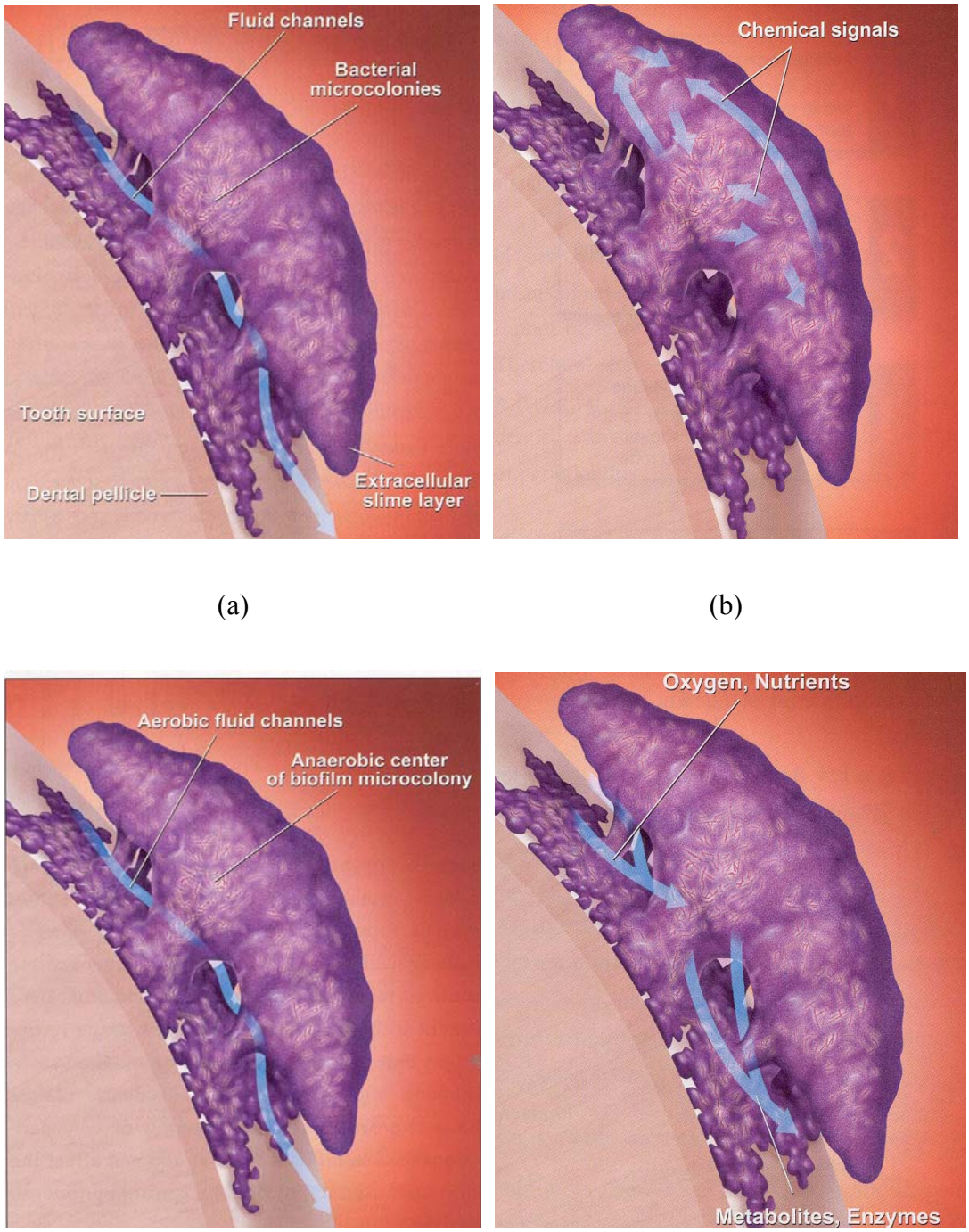
Figure 1: Schematic representation of steps in biofilm formation and its consequences (Prakash et al., 2003)

Bacterial biofilms have a complicated hierarchical organization and not a continuous monolayer surface deposit. The three dimensional structures of biofilms contain voids and channels for the passage and circulation of water and nutrients. These voids and channels also help in stabilizing the hydrodynamic equilibrium within the film thereby ensuring nutrient distribution and also maintain the heterogeneity of the microenvironment and rigidity of the biofilm structure. As the film grows in mass and

volume, the microclimate in various parts of the film becomes more and more differentiated. The interior of the film become secluded from the external environment leading to the development of anaerobic conditions within the film. Nutrient supply within the film is also depleted. As a result the bacteria residing deep within the film express a different set of genes than those having access to oxygen and nutrients in external environment. Most of the early colonizers are anaerobic forms. There exists a microenvironment difference for each cell and a differential growth rate is observed creating a gradient of metabolites, wastes and morphogenesis.

The human mouth is a reservoir of commensal microorganisms and as per the present knowledge houses more than 700 identified bacterial species (Paster et al., 2001; Aas et al., 2005; Paster et al., 2006) (Fig 2.). Initially thought to be free living, these bacteria are now known to form one of the most complex microbial communities of the human body. The different species of bacteria interact with each other to form the complex biofilm called the Dental plaque. Since saliva serves as a convenient supporter of bacterial growth, bacteria start colonizing the mouth of a new born right from birth. These species are called the early colonizers followed by the late colonizers. Dental plaques are usually formed by the normal oral microflora and are required for maintenance of both oral health and oral diseased condition. The study of dental plaque biofilm is important since the most common oral diseases including dental caries, gingivitis and chronic periodontis are caused the commensals and not by classic pathogens. Formation of dental plaque is a natural phenomenon responsible for maintaining normal healthy conditions of teeth. However, any ecologically driven imbalance in biofilm microenvironment can lead to severe diseased condition. For example, Caries, the most common of all oral diseases, is a result of low pH environment caused by microbial fermentation of carbohydrates which selects populations of acid-tolerant and acid-producing strains that in turn increase acid formation and may result in demineralization of the tooth structure (Fig 5.) Similarly in marginal periodontitis, accumulation of dental plaque enhances inflammation and increases the flow of gingival crevicular fluid. This environmental change may favor growth of various proteolytic bacteria, which outcompete other members of the micro-community to become

pathogenic by virtue of a numerical dominance (Gunnel Svensater & Gunnar Bergenholtz, 2004).



(a) (b)

(c) (d)

Figure 2: a) Three dimensional structure of a mature Dental plaque. b) Chemical signals used by bacteria to communicate within the film, c) Illustration showing aerobic

environment at the edges and anaerobic environment at the center of biofilm, d) Fluid channels facilitating nutrient and oxygen uptake within a biofilm.

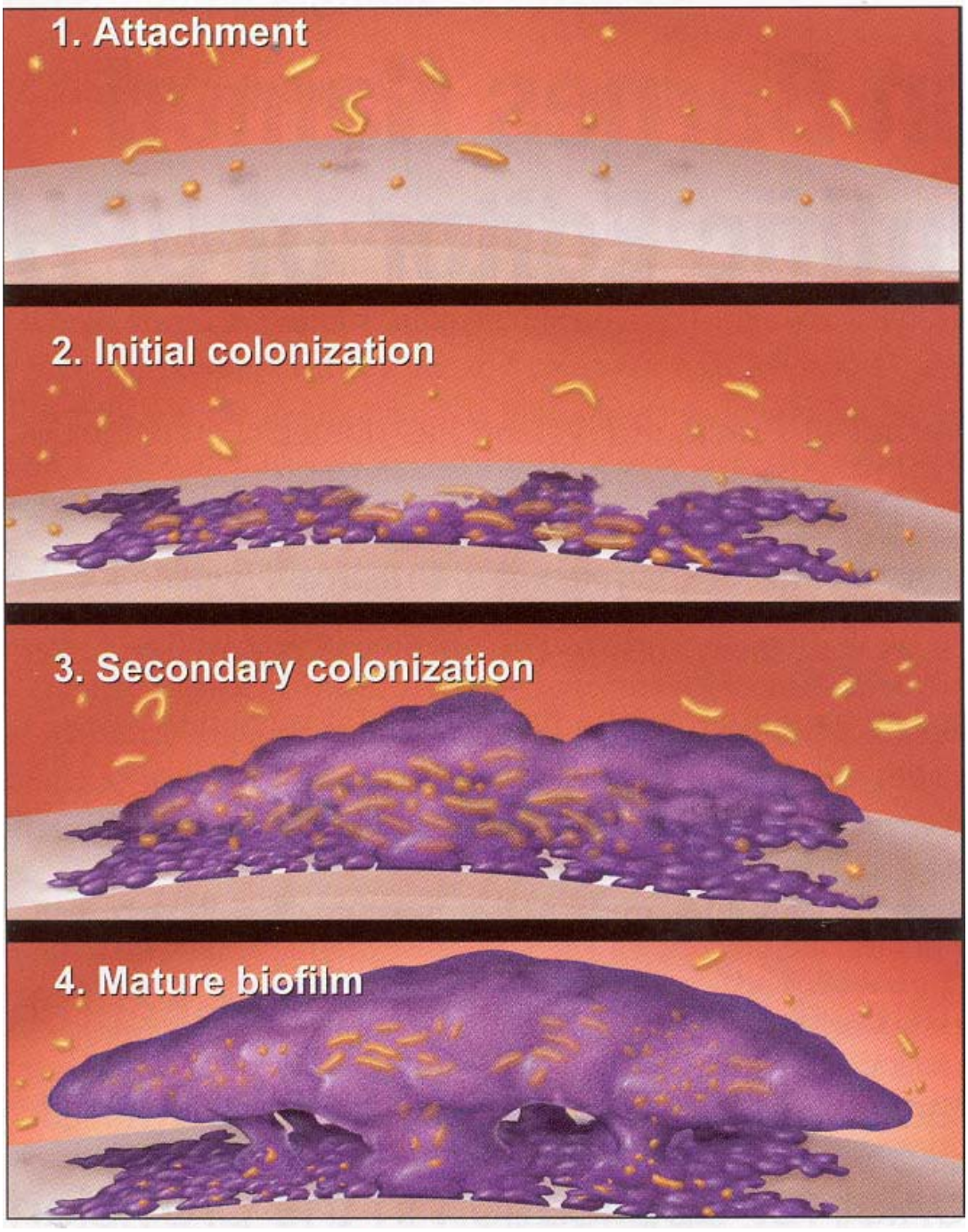


Figure 3: Pattern of biofilm development in dental plaque

In order to study the events involved in transformation from planktonic to colonial mode of existence, the interaction among different members of the biofilm community and the establishment of dental plaque biofilm using classical approach, the present project work has been undertaken. The study also aims at evaluation of stress response on development of dental plaque biofilm and qualitative and quantitative analysis of the EPS matrix which would be helpful in devising ways to prevent and disrupt these biofilms for prevention of periodontic diseases. An attempt has been made to study the kinetics of film formation, quorum sensing among the members of the film and chemotactic response shown by the bacterial species.

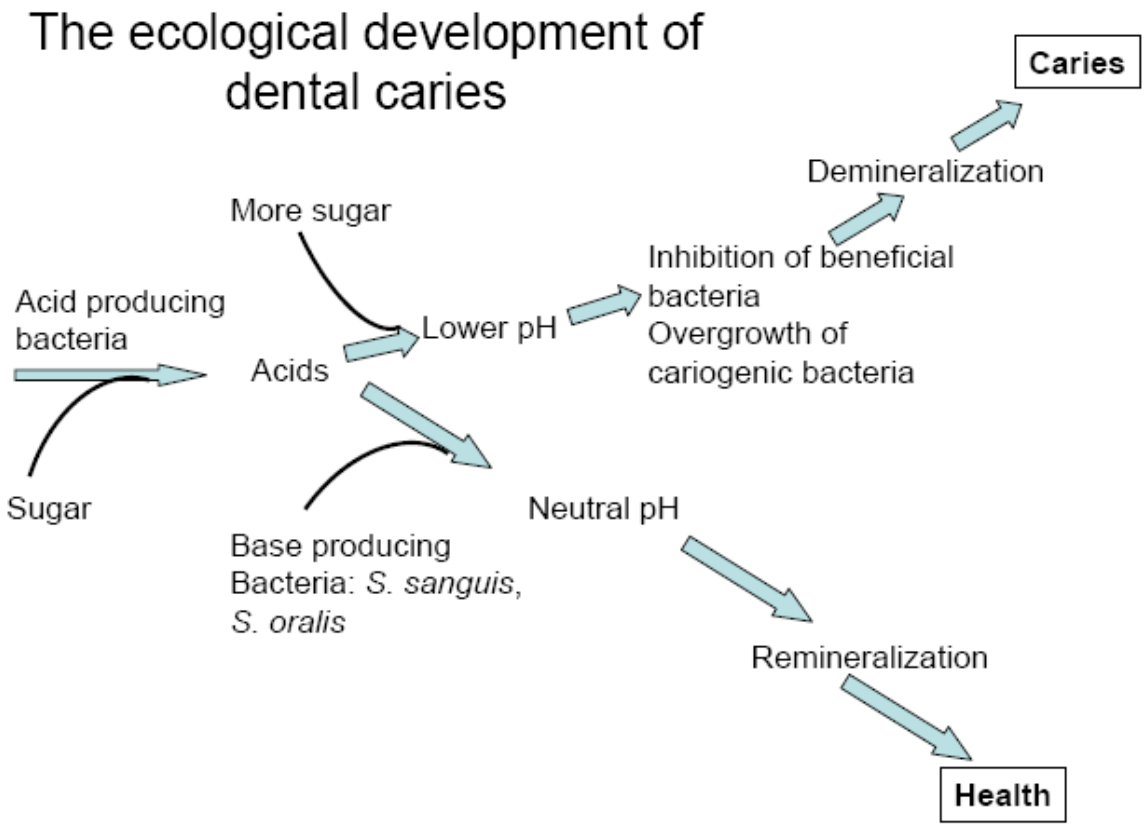


Figure 4: Schematic representation of ecological development of dental carries.

I. REVIEW OF LITRETURE

The discovery of biofilms can be credited to Antony van Leeuwenhoek who first observed tiny animalcules on the tooth surface using his simple microscope somewhere around 1680. Remarkable work was done by Characklis on bacterial slimes in water flow systems revealing their tenacious nature and resistance to disinfectants and biocides (Heukelekian et al., 1940) and by Costerton who coined the term biofilm in 1978 (Characklis, 1973). Charalicks put forward the theory of biofilm formation which involves three main processes including colonization, i.e., attachment of cells to the surface, growth of the attached cells into mature biofilms and erosion or sloughing, i.e., detachment of cells from the film.

The formation of biofilm follows specific mechanism of initial colonization on the surface, microcolony formation, development of organized three dimensional community structures and detachment from the surface.

Attachment is often favored by the presence of external structures on the bacterial surface e.g.: pilli, fimbrae or flagellum. As the bacteria approaches the surface its motility is slowed down and it forms a transient bond with the surface, a process called conditioning. Surface attached polysaccharides and proteins also play a major role in this process. Flow velocity, temperature and nutrient concentration are important factors governing the phenomenon (Costerton et al., 1999). After attaching to the surface the bacteria start producing signaling molecules which stabilize the film and attract other microorganisms to form microcolonies. As signaling molecules reach a threshold concentration the formation of mature biofilm takes place and there is switching on of a different set of genes which bring about further events of biofilm formation. The new set of genes encode for the production of extracellular matrix within which the colony is embedded. The matured biofilm has three dimensional structures with many voids and water channels meant for nutrient distribution and maintenance of hydrodynamic equilibrium. After complete maturation of a biofilm structure some cells continuously keep detaching from the biofilm surface either because of development of anaerobic conditions within the biofilm with increase in thickness of the film or because some of the cells die and some lose their capacity to produce EPS. Polysaccharidase enzymes

specific for the EPS of different organisms may possibly be produced during different phases of biofilm growth of the organisms and contribute to detachment (Boyd et al., 1994).

Dental plaque refers to the diverse microbial community found on the tooth surface, embedded in a matrix of polymers of bacterial and salivary origin. It develops naturally on teeth, and forms part of the defence systems of the host by helping to prevent colonisation of enamel by exogenous pathogenic microorganisms. The formation of dental plaque has long been associated to etiology of periodontal diseases (Black, 1900). In the past decades plaque structures, i.e.; the spatial arrangement of biofilm components have been studied using electron microscopes. The structural analysis of biofilm strongly depends on microscopic techniques. However most of the microscopic techniques require preparation of samples involving dehydrating, embedding etc. As a result the soft three dimensional structure of the film with voids and channels collapse and the film is viewed as a flattened structure (Stewart et al., 1995). Introduction of Confocal Scaning Laser microscope has revealed the three dimensional structure of the biofilm community. The CSLM images of undisturbed biofilms have shown biomass clustures separated by interstitial voids (de Beer et al., 1994).

Of clinical relevance is the fact that biofilms are less susceptible to antimicrobial agents, while microbial communities can display enhanced pathogenicity, i.e., pathogenic synergism (Marsh, 2004). The structure of the plaque biofilm might restrict the penetration of antimicrobial agents, while bacteria growing on a surface grow slowly and display a novel phenotype, one consequence of which is a reduced sensitivity to inhibitors (Gilbert et al., 2002). Plaque is natural and contributes to the normal development of the physiology and defenses of the host. Similar to other biofilm structures, the formation of biofilm follows an ordered sequence of events which includes attachment, growth, removal and reattachment .It is a continuous and dynamic process.

Steps recognized in the formation of dental plaque constitue (Fig 5):

1. Initial attachment of bacteria begins with pellicle formation which is a thin coating of salivary proteins attached to the tooth surface. The absorption of

salivary proteins and glycoproteins, together with some bacterial molecules, to the tooth surface to form this conditioning film called the acquired pellicle.

2. Following formation of the acquired pellicle, long-range, i.e., >50nm, non-specific interaction of microbial cell surfaces with the acquired pellicle via van der Waals attractive forces is established.
3. Shorter-range interactions within 10 -20 nm is dominant in which the interplay of van der Waals attraction forces and electrostatic repulsion produces a weak area of attraction that can result in reversible adhesion to the surface.
4. Irreversible adhesion occurs when specific inter-molecular interactions take place between adhesins on the cell surface and receptors in the acquired pellicle.
5. Secondary or late-colonizers attach to primary colonisers**,** by specific inter-molecular interactions.
6. Cell division of the attached cells to produce confluent growth, and a bioflim.

The predominant bacterial species include *Streptococcus*, *actinomyces*, anaerobic gram positive rods, *Nesseria*, *Veilonella,* Anaerobic gram negative rods etc. A heterogenous and a colonial type of sub-structure can be observed in sections of smooth surface plaque. The heterogeneous type is associated with pallisaded regions where filaments and cocci appear to be aligned in parallel at right angles to the enamel surface. Micro-colonies, presumably of single populations, have also been observed. In addition, horizontal stratification has been described. The early stages of development results in a condensed layer of apparently a limited number of bacterial types. From 7 to 14 days, the bulk layer forms which shows less orientation but a higher morphological diversity. This layering has been attributed directly to bacterial succession. In mature plaque, organisms have been seen in direct contact with the enamel due to enzymatic attack on the pellicle. In a dental plaque biofilm the microorganisms are in close proximity to one another and interact as a consequence. Microbial metabolism within the plaque produces gradients in factors affecting the growth of other species, including the depletion of essential nutrients with the simultaneous accumulation of toxic or inhibitory byproducts. These gradients lead to the development of vertical and horizontal stratifications within the plaque biofilm.

Such environmental heterogeneity enables organisms with widely differing requirements to grow, and ensures the co-existence of species that would be incompatible with one another in a homogeneous habitat. Beneficial interactions include the concerted action of two or more species to metabolise host macromolecules, such as mucin while individual species are unable to catabolise such molecules. Antagonistic interactions include the production of inhibitory substances such as bacteriocins, H_2O_2, and organic acids.

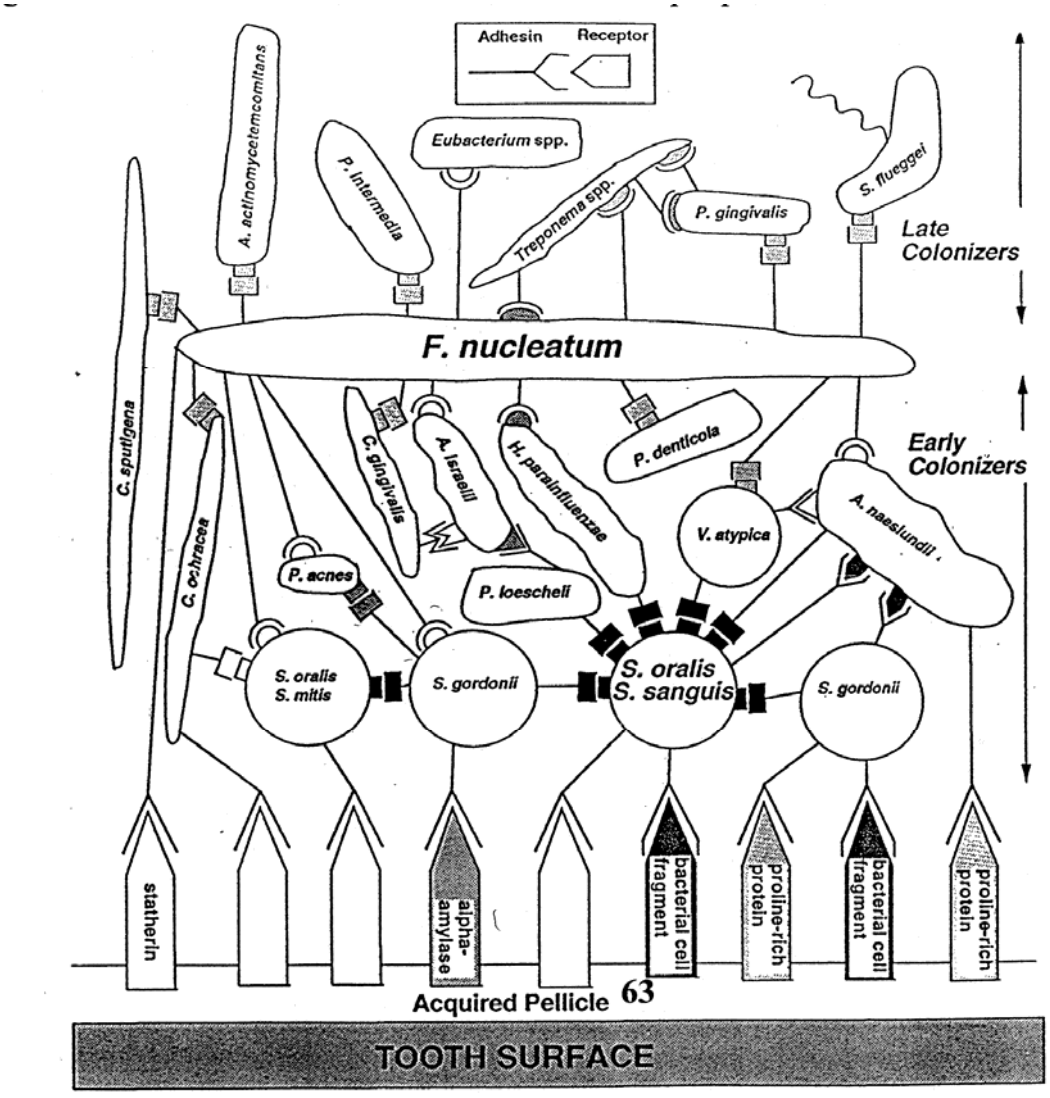


Figure 5: Intermolecular interactions involved in biofilm development

Early colonisers of the tooth surface are mainly *Neisseria* spp. and streptococci. The growth and metabolism of these pioneer species changes local environmental conditions (e.g. Eh, pH, coaggregation, substrate availability) thereby enabling more fastidious organisms to colonise, e.g. obligate anaerobes tend to be late colonisers in plaque, only able to grow once favourable gradients in O_2 or Eh have developed in the biofilm. The microbial homeostasis is maintained due to dynamic balance between the resident members of the microbial community. As the homeostasis breaks down, the site becomes susceptible to diseases. High intake of fermentable sugar in the diet lowers the pH in plaque inhibiting the growth species associated with dental health, giving rise to conditions conducive to growth of the highly acidogenic and aciduric species, like mutans streptococci and lactobacilli, associated with dental caries. In periodontal diseases, there is change in composition of the plaque microflora to a more proteolytic Gram negative anaerobic community**,** which can induce damage to tissues either indirectly via the “side-effects" of an inflammatory host response or directly by production of proteases, cytotoxins and other virulence factors.

Periodontal infections not only affect oral health but also have a pronounced influence on general health of an individual since mouth is the most convenient port of entry for pathogens, mouth being the first part of the alimentary canal. Oral infections may affect the course and pathogenesis of a number of systemic diseases including Diabetes, Heart disease, premature birth (low-birth-weight), Lung disease etc (Fig. 6). A number of possible causes for spreading of such infections have been suggested .Some of these possible mechanisms include:

- Spread of infection from the oral cavity as a result of transient bacteremia.
- Injury from the circulating oral microbial toxins
- Inflammation caused by bacterial component-virulence determinants
- Subgingival dental plaque as reservoirs of gram negative bacteria, resulting in inflammatory mediator

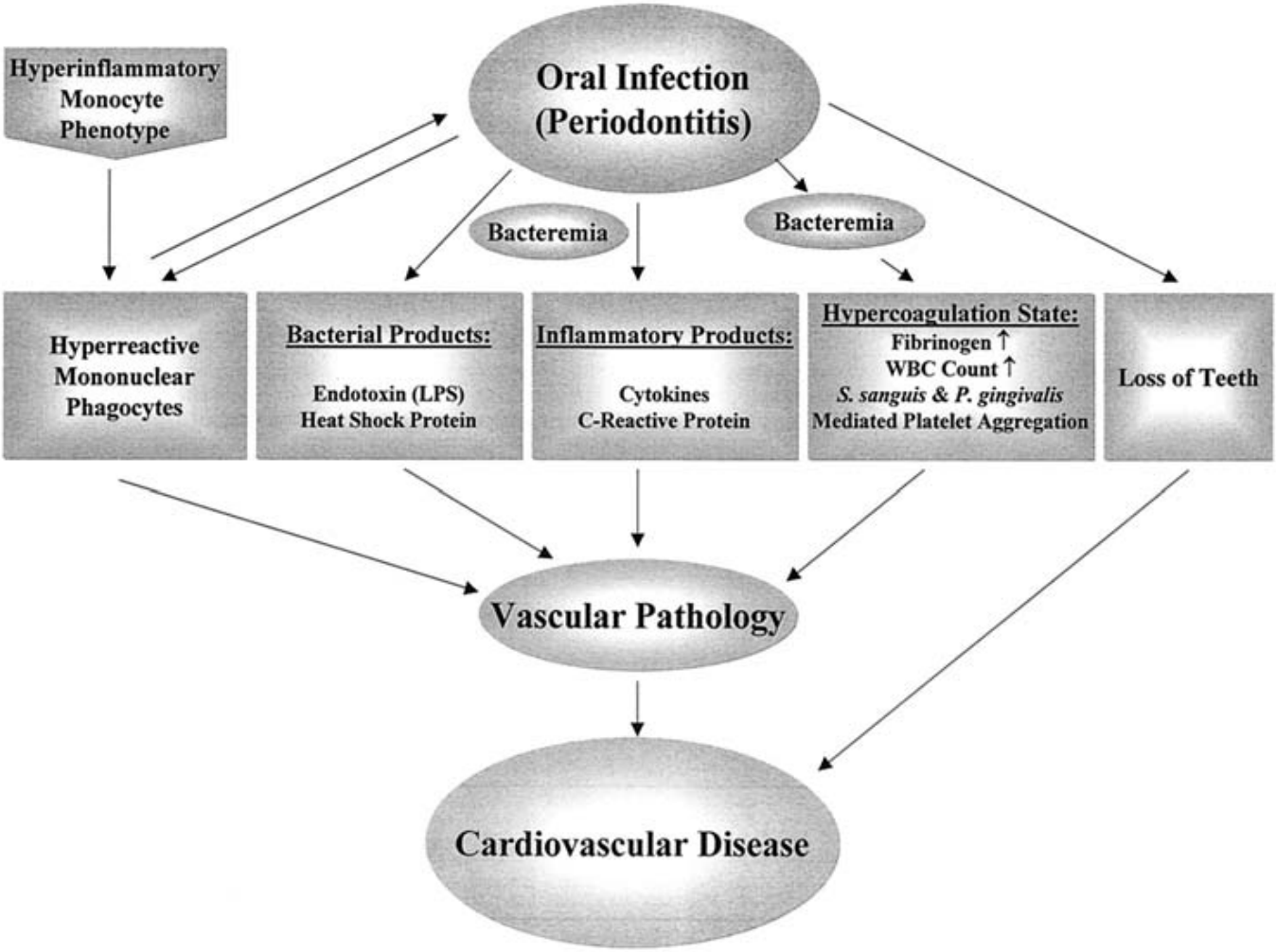


Figure 6: Chart showing interrelationship of periodontal infections with other systemic infections (Dental conference I, Seok-Woo Lee, DDS, MS, PhD Division of Periodontics, School of dental and oral surgery, Coulombia University)

II. OBJECTIVES

Dental plaque biofilms are very heterogeneous in structure. Dense mushroom-like structures originating from the enamel surface, interspersed with bacteria-free channels used as diffusion pathways filled with an extracellular polysaccharide (EPS) matrix produced by the bacteria. They generally live under nutrient limitation and often in a dormant state. Such "sleepy" bacteria respond differently to antibiotics and antimicrobials than expected from laboratory studies. In addition, it has been found that many therapeutic agents bind to the biofilm EPS matrix before they even reach the bacteria, and are thereby inactivated. Taking these facts into account the present study was undertaken with the following objectives:

1. Isolation and chacterization of dental plaque forming bacteria.
2. Biochemical identification of bacterial strains
3. In vitro screening for biofilm formation and physicochemical studies of biofilm formation.
4. Evaluation of response to following stress parameters :

    i) Sugars
    ii) Salinity
    iii) Temperature
    iv) pH
    v) Metals
    vi) Antibiotics

5. Study of chemotactic response to different sugars and Xenobiotics
6. Planktonic growth kinetics and Kinetics of biofilm formation
7. Detection extraction and analysis of Quorum Sensing signal molecules in dental plaque forming bacteria.

III. MATERIALS AND METHODS

Sample collection

Bacterial sample was collected from oral cavity by swabbing across the gingival and subgingival region as well as from the roof and floor of the buccal cavity. Representative samples were collected from three persons and the samples were inoculated in Nutrient broth and the sample was prepared for enumeration of viable cell count.

Enumeration of viable cell count

The overnight broth culture was serially diluted with autoclaved distilled water upto 10^{-6} dillution and 100 µl of each dilution was spreaded on to Nutrient agar plates and incubated overnight at 37ºC .After in incubation period of 12 -18 hours the number of viable colonies were counted using total viable plate count method.(Prescott and Harley, 2002).

C.F.U/mL original sample = No. of colonies /plate x (1/mL aliquot plated) x dilution factor

Isolation and screening of biofilm forming bacteria:

10 colonies with visually distinguishable morphologies were randomly selected and isolated by directly streaking on Nutrient agar plates and incubated for another 12-18 hours. The isolated colonies were then restreaked after incubation onto nutrient agar plates to obtain pure cultures. The viability of the isolated cultures were checked in Lauria Bretani broth and those found to be viable were screened for biofilm formation. Primary biofilm screening was done using tube staining assay (Christensen *et al*., 1982) as well as the microtiter plate biofilm assay (Mack et al., 1994; O'Toole et al., 1999).

The Tube assay:

Qualitative assessment of biofilm formation was determined by the tube staining assay. LB broth (5mL) was inoculated with 10µl of overnight culture broth and incubated for 24, 48 and 72 hours at 37°C. The tubes were decanted and washed with PBS (pH 7.3)

and dried. Dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation.

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate or 3-strong Experiments were performed in triplicate and repeated three times. (Mathur et al., 2006).In one set the media was inoculated and kept static for the entire incubation period and in another set the media was replaced with fresh media every 24 hours.

The Microtitre plate assay:

The microtitre plate assay is the most widely used and is considered as standard test for detection of biofilm formation. The isolates were screened for their ability to form biofilm by microtitre plate method with modification in duration of incubation period. Since composition of media is known to affect biofilm formation, the cultures were screened in three different media ie; Nutrient broth, LB broth and Basal media supplemented with glucose. Isolates from fresh agar plates were inoculated in respective media and incubated for 18 hour at 37^{o}C in stationary condition and diluted 100 times with fresh medium. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates (Tarson, Kolkata, India) wells were filled with 200µl aliquots of the diluted cultures and only broth was used as control.

The tissue culture plates were incubated for 24 hours, 48 hours and 72 hours at 37°C. After incubation content of each well was gently removed by tapping the plates. The wells were washed with phosphate buffer saline (PBS pH 7.2) to remove free-floating ‘planktonic’ bacteria. Biofilms formed by adherent ‘sessile’ bacteria were stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with 95% ethanol and plates were kept for drying. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader at wavelength of 570 nm. These OD values were considered as an index of bacteria adhering to surface and forming biofilms. The experiment was performed in triplicate and repeated three times, the data

was then averaged. The mean OD value obtained was compared with that of empty wells. The isolated bacterial strains which were found to be biofilm forming were preserved in nutrient agar slants and cryovials refrigerated at 4ºC for further use. The isolated bacteria were then gram stained to determine the morphology and cell wall characteristics. The gram negative isolates were identified by standard biochemical tests (Collins and Lyne, 1970; Hansen and Sorheim, 1991) as per the requirements of bacterial identification software PIBWin (Byrant, 2004) and Bergey's manual of determination bacteriology.

Characterization of bacterial strains:

Physical characterization:

i) Gram staining**:** The diluted suspensions of the bacteria were smeared on clean slides, air dried, heat fixed by passing over a flame for 2 to 3 times. The slides, were flooded with crystal violet solution for one minute, washed with water and flooded with Gram's iodine for one minute. The slide were washed with water and decolorized with 95% ethyl alcohol dropped from a dropping bottle until no violet colour was visible from drain off solution. The slides were washed with water and counter stained with safranin stain for about 30 second and washed with water. The slides were air dried and examined under a microscope using 100x objectives using a daylight filter. Cells were then identified by the colour observed purple for Gram positive and pink or red for Gram negative cells.

ii) Colony morphology: Shape, size, colour, elevation and margin of colony and appearance are observed in overnight plate culture on Nutrient agar media and noted down.

iii) Cell morphology: The gram stained cells were viewed under light microscope under 100x oil emmersion to determine the shape and size of the cells.

iv) Motility test: It is used to check the ability of bacteria to migrate away from the line of inoculation. The bacteria was inoculated into SIM or motility media, i.e, mannitol agar with a needle by stabbing the culture in a straight line and was observed after 24-48 hours incubation. If the test organism migrates away from the line of inoculation, the bacteria is motile.

Biochemical characterization:

Himedia Rapid Biochemical Identification kit, *Enterobacteriaceae* Identification Kit [KB003 Hi25®] was used. KB003 is the comprehensive test system used for identification of gram negative *Enterobacteriaceae* species. Single well isolated colony was picked up and inoculated in 10 ml NA broth and incubated at 37ºC for 24 hours. Oxidase test was performed on organism to be tested to differentiate *Enterobacteriaceae* from other gram negative rods using the Oxidase disc provided with the kit. Kit was opened aseptically and sealing tape was peeled off. Each well was inoculated with 50 µl of the above inoculums by surface inoculation method and kept for inoculation at 35- 37°C for 18-24 hours. At the end of the incubation period, a series of reagents were added in designated wells as per manufacturer's specifications to carry out different biochemical tests.

Evaluation of stress response

i) Sugar stress:

Stock solution of five sugars ie; glucose, sucrose, arabinose, fructose and Xylose having concentration 0.1 g/ml was prepared in autoclaved water and sterilized by filtering through 0.22µm pore size membrane filters and stored at 4ºC.Stress response was studied by incorporating different sugars in basal media in which the bacterial strains were cultured 1 mg/ml of sugar in basal media was prepared for each sugar and 1980µl of media was inoculated with 20 µl of overnight broth culture of the bacterial strains. 200µl of the same concentration of inoculum was inoculated in 96 well microtitre plates and was incubated at 37ºC for 72 hours. After incubation, the content of the plates were removed by tapping and was rinsed with Phosphate Buffer Saline (PBS) to remove the planktonic cells. The attached biofilm was stained with 10 times diluted Grams Crystal Violet for 15 minutes followed by destaining with 95% ethyl alcohol. The alcohol was rinsed was allowed to dry. The OD of the adherent biofilm was taken using a micro ELISA autoreader (Perkin Elmer) at 570nm.The tubes were stained in similar manner and results obtained with different sugars were compared.

ii) Salinity:

Salt stock of concentration 1mg/ml was prepared in autoclaved water and filter sterilized using 0.22µm membrane filters. The salt stock was serially diluted in LB broth to obtain a concentration of 0.2, 0.4, 0.6, 0.8 and 1 mg/ml. 1980µl of media having different salinity were inoculated with 20 µl of overnight broth culture of bacteria. 200µl of same inoculum concentration was inoculated into 96 well microtitre plates and incubated at 37ºC for 72 hours. After incubation the tubes and plates were stained and analyzed as described above.

iii) Temperature stress:

1980µl of LB broth was inoculated with 20µl of overnight bacterial culture in three sets and 200µl of 1 in 100 dilution of overnight culture was inoculated onto 96 well microtitre plates. The tubes and tissue culture plates were incubated at three different temperatures for 72 hours and at the end of incubation the tubes and plates were stained and analyzed as described above.

iv) pH stress:

The pH of media was adjusted to 6.4, 7.4, and 8.4 by addition of acid and base respectively. The media was inoculated in plates and tubes as above and incubated for 72 hours. After incubation the tubes and microtitre plates were stained and analyzed as above.

v) Metal stress:

Metal salt stock of six metals Mg, Cd, Ca, Zn, Fe and Cu having concentration 0.2mg/ml was prepared in autoclaved water and filtered through 0.22µm membrane filters and stored at 4ºC. The metal stock was diluted in LB broth so as to obtain concentration of 10, 20, 40 and 80 ppm. The media was inoculated with overnight broth culture as incubated for 72 hours followed by staining and analysis as described above.

Antibiotic stress:

Biofilm formation was tested in presence 0.2, 0.4, 0.6, 0.8 and 1µg/ml of Penicillin, Chloramphenicol, Tetracyclin, and Rifampicin at 37ºC.

Chemotaxis:

Chemotactic response of the isolated bacterial strains to different sugars and xenobiotics by the plug punching method. 5 ml of LB broth was inoculated with 20µl of overnight culture and incubated at 37ºC to attain the mid log phase (2-3 hours). 25 ml of liquid PBS soft agar held at 42ºC was seeded with 25 ml of actively growing culture, was shaked well and poured into petridish and allowed to cool down and set. To prepare plugs, 0.5% of the compound to be screened was added to liquid PBS hard agar held at 50ºC, poured into petridish and allowed to cool and set. Using gel puncher, plugs of compound containing hard agar was punched out. Using a sterile forecep plugs of the agar containing compounds to be tested are picked up and pushed into the organism containing soft agar plate. The plates were then incubated upright at 37ºC for 12-18 hours. After incubation chemotaxis rings are checked around the agar plugs which indicates that the organism is capable of using the compound tested.

Planktonic growth kinetics:

50 ml of LB broth was inoculated with 5µl of overnight broth culture of the test organism and initial OD was taken at 630 nm. The culture was incubated at 37ºC and 180rpm and the OD was measured at an interval of every half an hour. A graph of absorbance vs time was plotted to obtain the growth kinetics of the organism.

Kinetics of biofilm formation:

100µl each of 1:100 dilution of overnight broth cuture of the test organisms were inoculated into 5 sets of 96 well microtitre plates and the absorbance was read out using ELISA auto reader at 570 nm at an interval of every 2 days following similar staining procedures as described above. The OD values at different tie

time interval were plotted against time period to obtain the kinetics of biofilm formation.

Qualitative and Quantitative analysis of EPS matrix:

EPS extraction: Biofilm samples were thawed on ice and centrifuged at 15000× g for 20 minutes. The biofilm pallets were resuspended in about 30 ml of cold sulfuric acid ie 0.2 M sulfuric acid, pH 1.1 and the biofilm matrix was broken using a glass homogenizer tube and pestle. The cell suspension was kept at 4ºCor 3 hours with occasional stirring before centrifugation at 15000 × g for 20 minutes. The resulting supernatant containing total EPS of both colloidal and capsular fractions was used for further analysis (Jiao et al, 2010).

Chemical composition: Fourier transform infrared spectroscopy was carried out with ethanol precipitated and dried EPS samples. EPS was precipitated using three volumes of 100% cold ethanol and incubated in ice for 2 hour. The precipitates were centrifuged at 17500 × g for 20 minutes at 4ºC and dried in an oven overnight at 50ºC .FTIR spectroscopy was performed .(Jiao et al, 2010).

The total protein concentration was measured by adding 12% Trichloroacetic acid to EPS solution and the mixture was incubated on ice for 30 minutes before centrifugation at 15,000×g for 20 minutes. The TCA precipitates were washed twice with 10 ml acetone and resuspended in 2ml of 2-N-morpholinoethanesulfuric acid (MES) buffer pH 5.0.The protein content was estimated using Bradford protein assay with BSA as the calibration standard (Jaio et al, 2010).

Total DNA content was estimated in EPS solution after extraction with 3 volumes of 100% cold ethanol. The mixture was incubated on ice for 2 hours before DNA was recovered by centrifuging at 17,500×g for 20 mins at 4ºC.The DNA content was measured by reading out the absorbance at 260nm

Total carbohydrate content was measured using modified phenol-sulfuric acid method with glucose standards (Dubois et al, 1951). 50 µl of EPS solution was mixed with 125µl of concentrated sulfuric acid. 25µl of 10% phenol was mixed

and the mixture was incubated in 95ºC water bath for 5 minutes. The mixture was cooled and absorbance was read out at 595 nm using a spectrophotometer.

IV. RESULTS AND DISCUSSION

Enumeration of Total viable cell count:

Total viable count was determined from selected plates having 30 to 300 colonies. (Table 1)

Total viable count was calculated from the formula

THB = No. of colonies × Dilution factor / Inoculum size in CFU/ ml

Table 1: Viable cell count

Sl.no.	Number of bacterial colonies	Dilution factor	THB(cfu/ml)
1.	280	10^{-2}	2.8×10^{5}
2.	200	10^{-3}	2.0×10^{5}
3.	160	10^{-4}	1.6×10^{5}

10 bacterial strains with observable difference in colony morphology were randomly selected from initial spread plate. (Fig 7.)

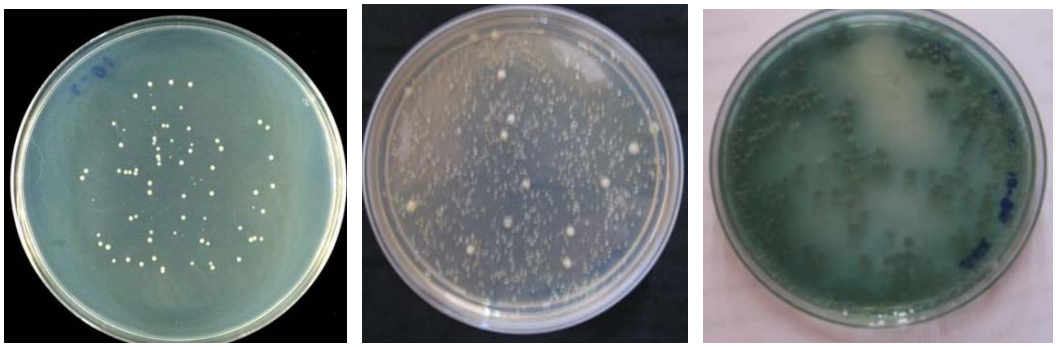


Figure 7 : Spread plate culture of dental plaque bacteria

Isolation of strains:

The 10 strains selected with observable difference in colony morphology were pure cultured by quadrant streaking. (Fig 8.)

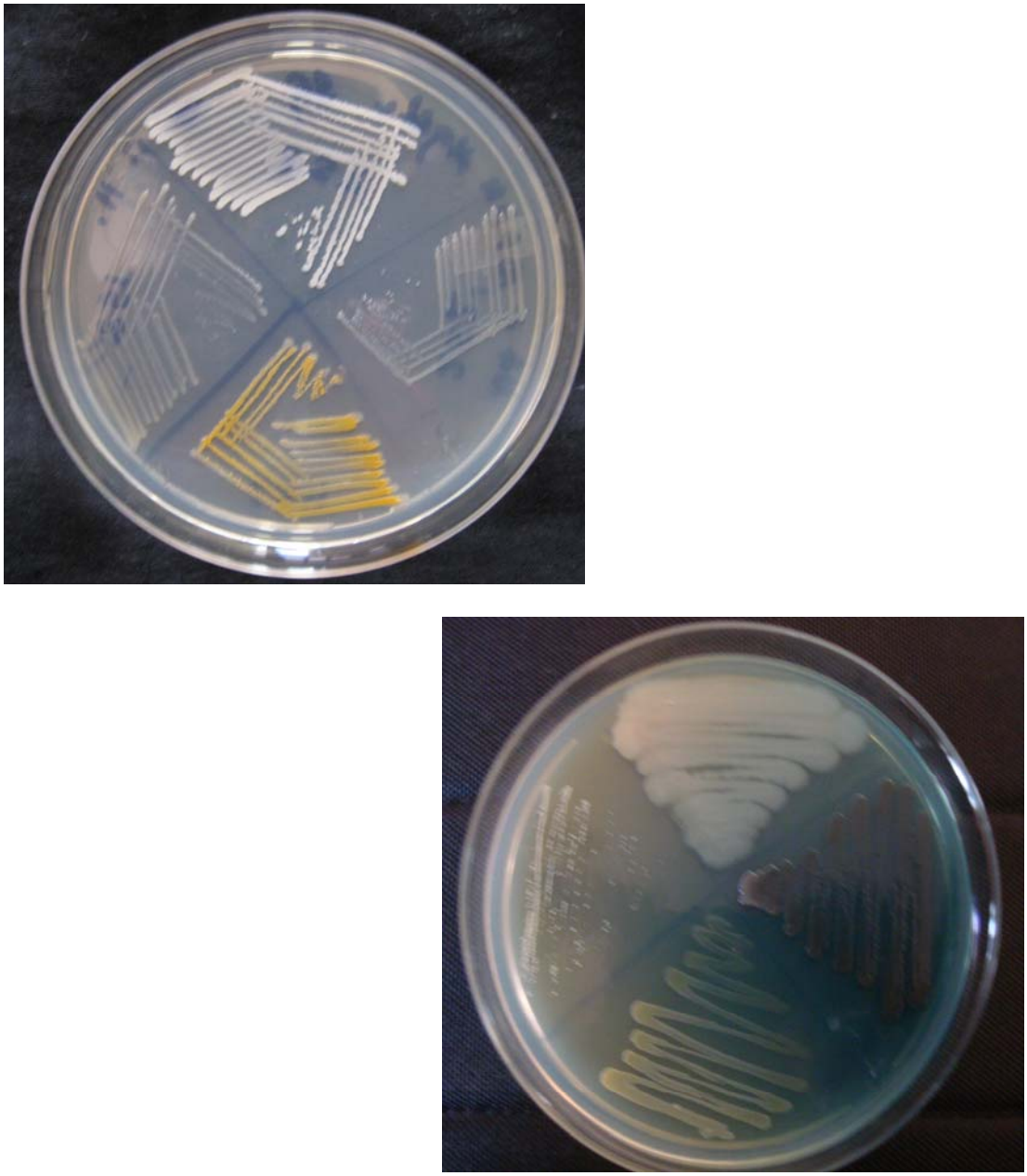


Figure 8: Pure cultures of the isolated strains

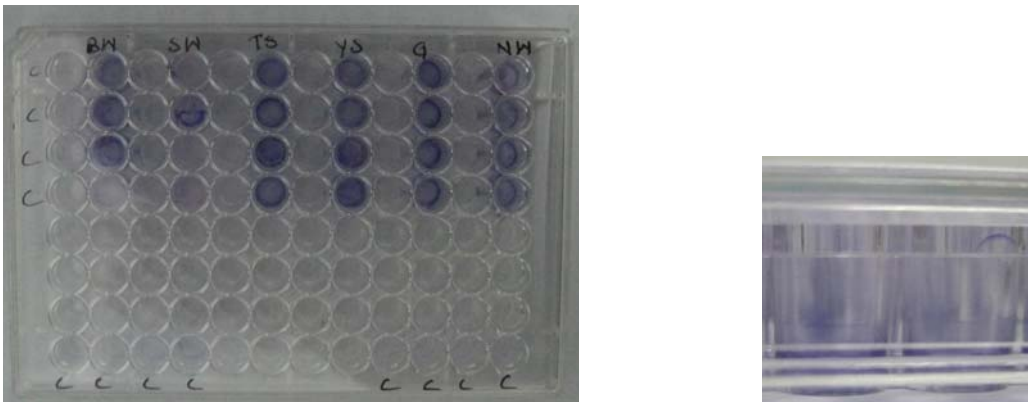
PRIMARY SCREENING FOR BIOFILM FORMATION

After primary screening, six strains were found positive for biofilm formation. They were named as BW, SW, TS, YS, G and NW. (Fig 9).

Tube assay: Tubes when stained with crystal violet showed biofilm attachment.

Figure 9: Stained tubes showing biofilm attachment

Microtitre plate assay: (Primary screening) The microtitre plate assay showed attachment at the bottom of the well. The samples were kept in alternative wells (2, 4, 6, 8, 10) and control was taken in well 1.(Fig 10)



(a) **(b)**

Figure 10: Three day biofilm screening of the isolated strains

Absorbance of 3 and 5 day biofilm screening are showed in Table 2 and 3

Table 2: ELISA reading of 3 day old biofilm

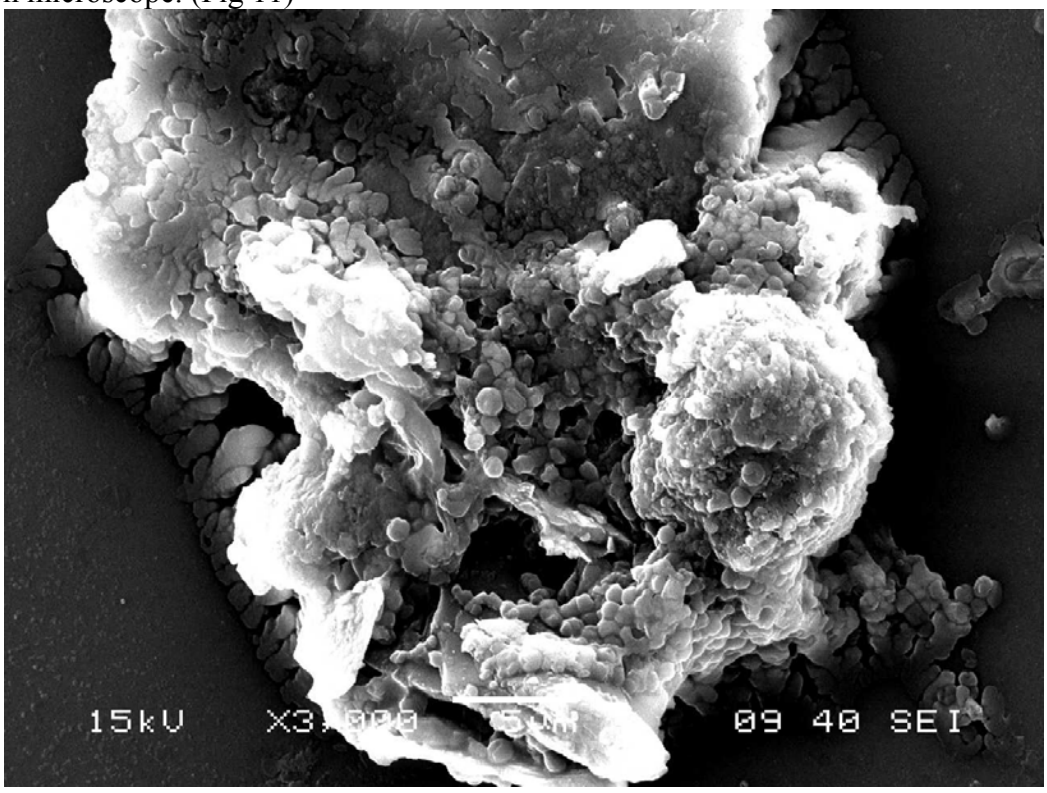
	1 (c)	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.40	0.444	0.04	0.047	0.041	0.044	0.04	0.047	0.041	0.054	0.039	0.056
B	0.04	0.047	0.041	0.045	0.04	0.044	0.04	0.049	0.04	0.052	0.04	0.058
C	0.39	0.444	0.038	0.045	0.039	0.041	0.039	0.048	0.041	0.055	0.04	0.057
D	0.041	0.445	0.039	0.047	0.039	0.042	0.039	0.049	0.039	0.055	0.039	0.056
E	0.039	0.04	0.04	0.04	0.039	0.04	0.04	0.04	0.041	0.04	0.039	0.04
F	0.04	0.039	0.039	0.041	0.039	0.04	0.039	0.039	0.039	0.039	0.04	0.041
G	0.04	0.039	0.039	0.04	0.039	0.04	0.04	0.04	0.039	0.04	0.04	0.04
H	0.039	0.04	0.039	0.04	0.04	0.039	0.04	0.04	0.04	0.04	0.041	0.04

Table 3: 5 day screening:

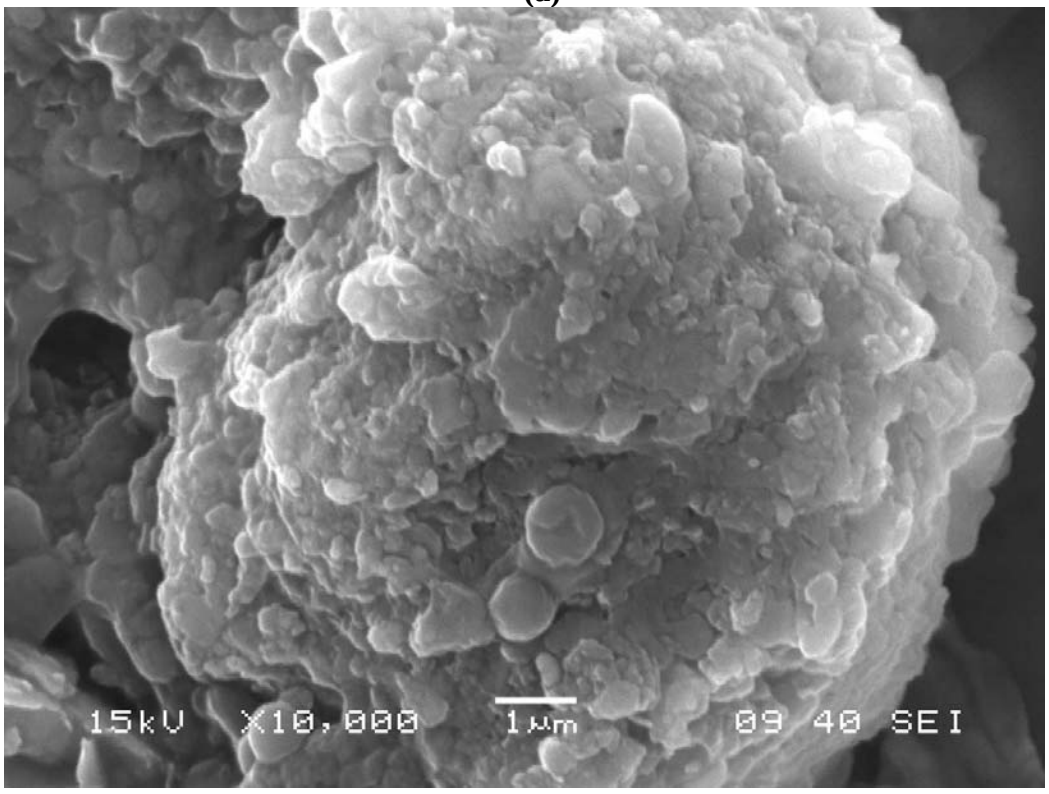
	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.6	0.04	0.278	0.041	0.222	0.042	0.122	0.041	0.444	0.04	0.051
B	0.04	0.06	0.04	0.079	0.04	0.057	0.041	0.065	0.041	0.024	0.04	0.416
C	0.041	0.077	0.04	0.072	0.04	0.079	0.041	0.076	0.041	0.096	0.04	0.077
D	0.041	0.048	0.039	0.054	0.04	0.083	0.04	0.789	0.041	0.098	0.04	0.876
E	0.041	0.04	0.039	0.04	0.039	0.04	0.039	0.04	0.041	0.04	0.04	0.04
F	0.041	0.04	0.04	0.041	0.041	0.041	0.041	0.041	0.041	0.04	0.04	0.04
G	0.04	0.041	0.04	0.04	0.04	0.041	0.04	0.04	0.041	0.04	0.04	0.04
H	0.04	0.04	0.039	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04

Wells in Grey: Showing intensity of biofilm attachment
Wells in Yellow: Control (Containing only media)

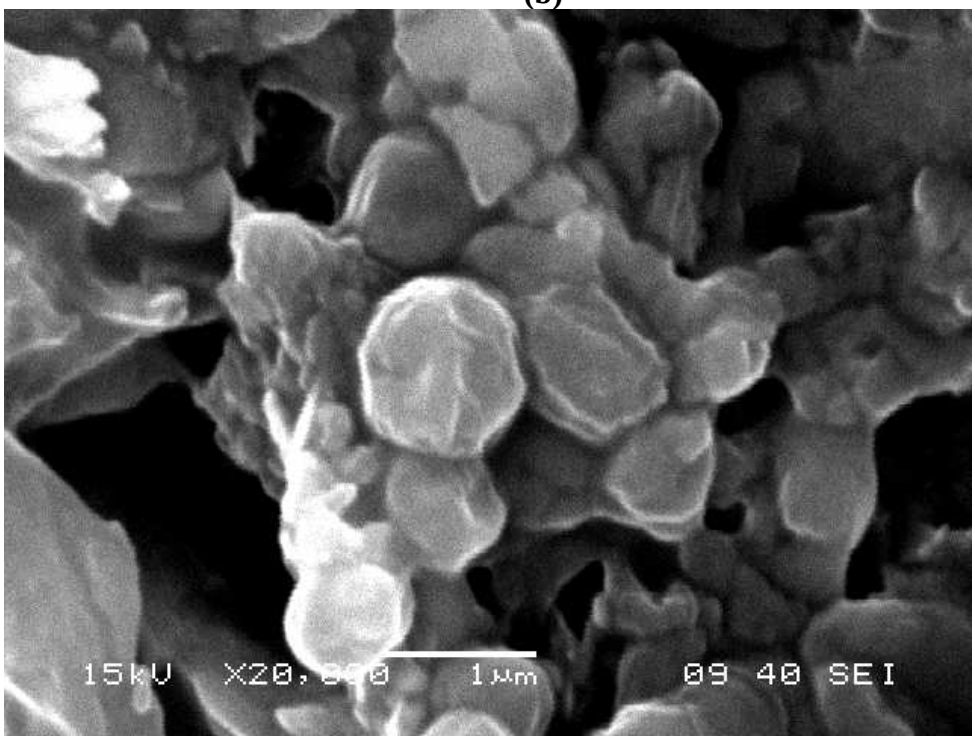
SEM analysis of biofilms formation: Biofilm formed was observed under Scanning electron microscope. (Fig 11)



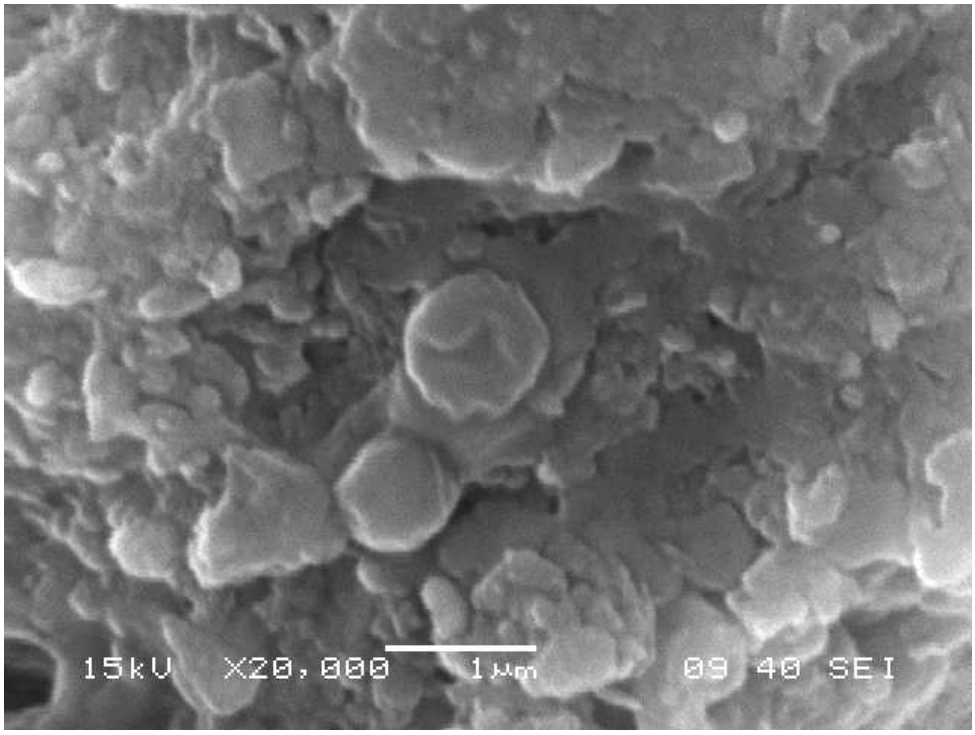
(a)



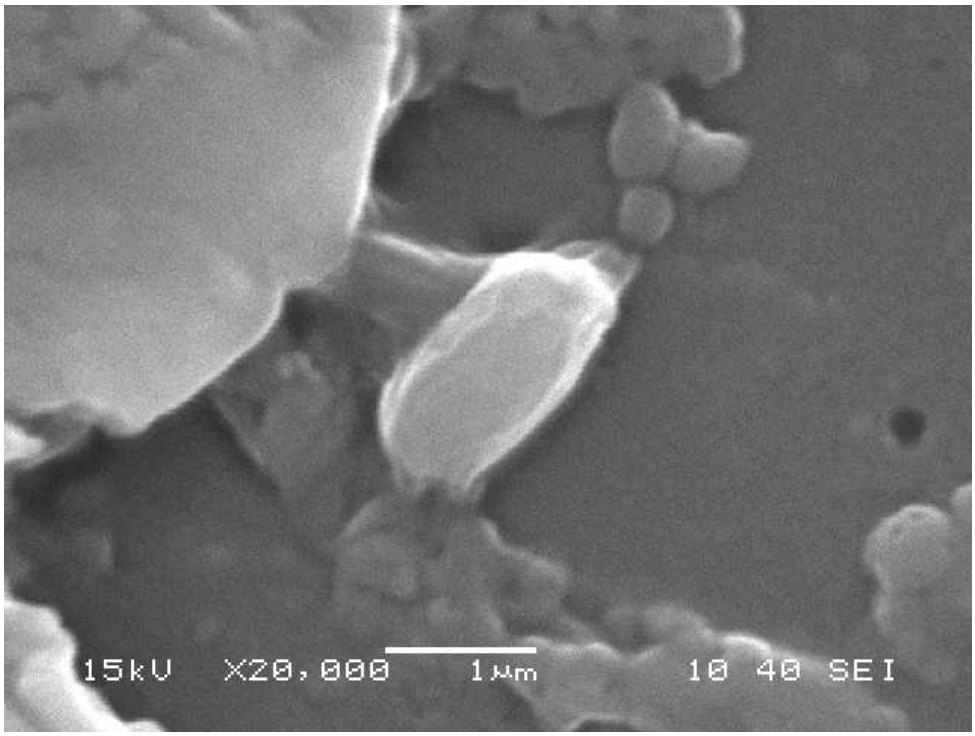
(b)



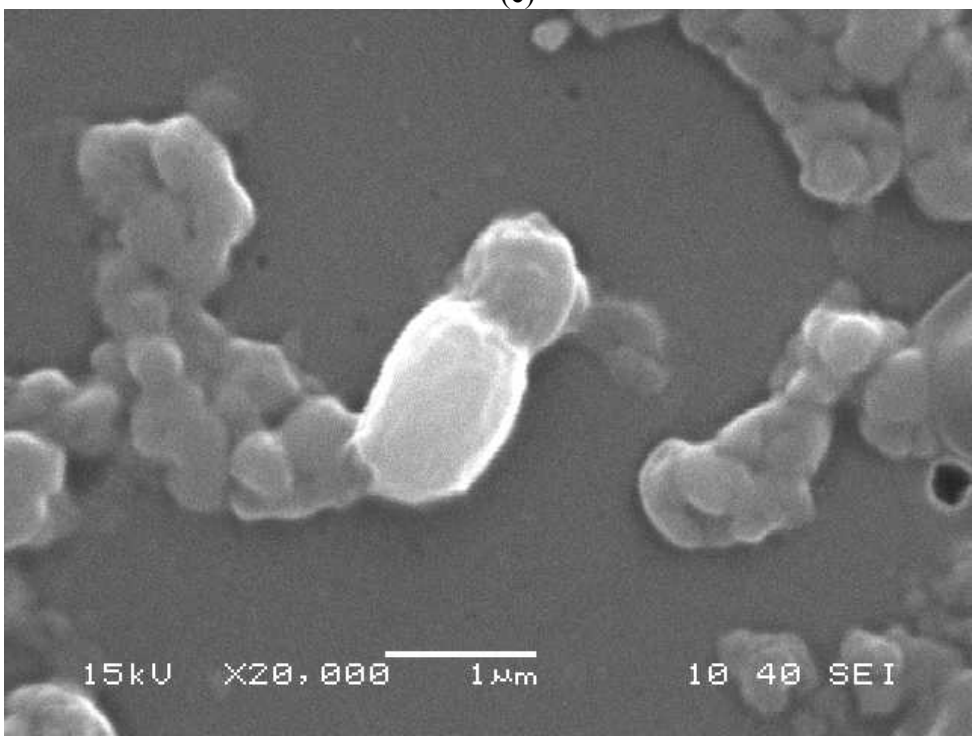
(c)



(d)



(e)



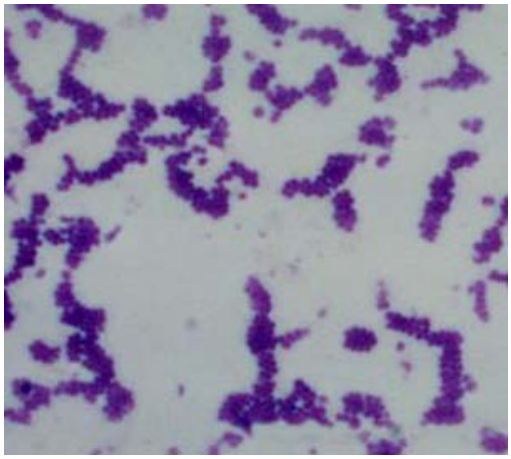
(f)

Figure 11: SEM pictures of (a-d) Biofilm formed by BW, (e-f) Biofilm formed by NW at different magnifications.

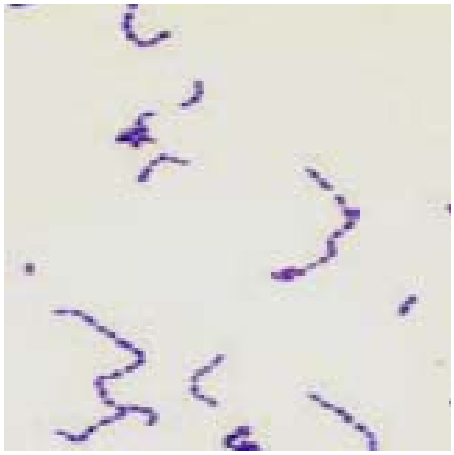
CHARACTERIZATION OF BACTERIAL STRAINS

Physical characterization:

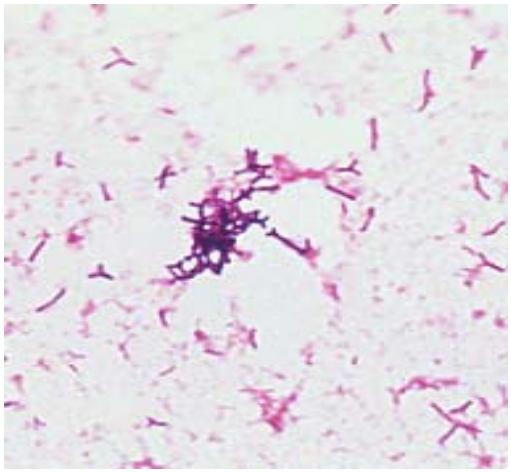
The isolated bacterial species BW, SW, TS, YS, G and NW were morphologically characterized by gram staining and were found be Gram +ve staphylococcus, Gram +ve staphylococcus, Gram +ve streptococci, Gram +ve streptococci, Gram –ve bacilli and Gram +ve bacilli respectively(Fig 12 a-e). Motility test for all bacterial strains were done on SIM media and all were found to be motile.(Fig 13)



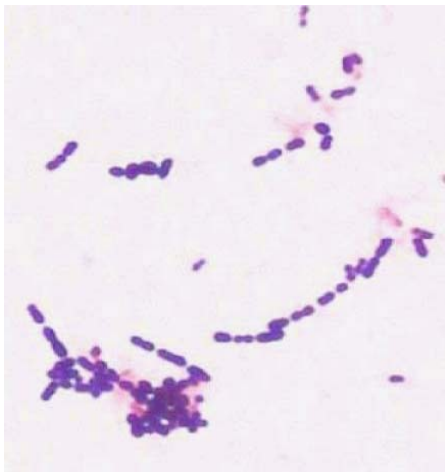
a) Staphylococcus aureus



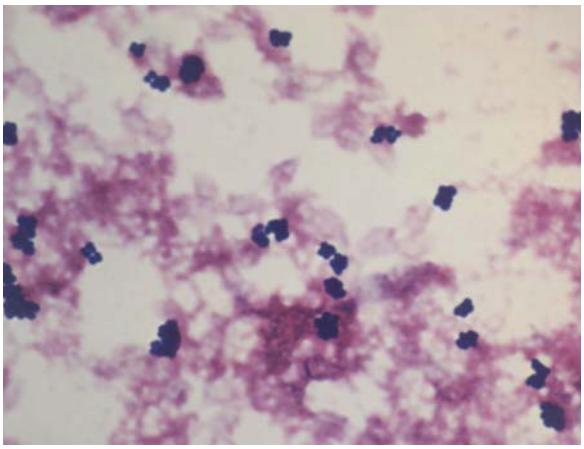
b) Streptococcus mitis



c) Bacillus subtilis



d) Streptococcus oralis



e) Staphylococcus salivarius

Figure 12 (a-e) Gram staining pictures of the isolated bacterial strains.

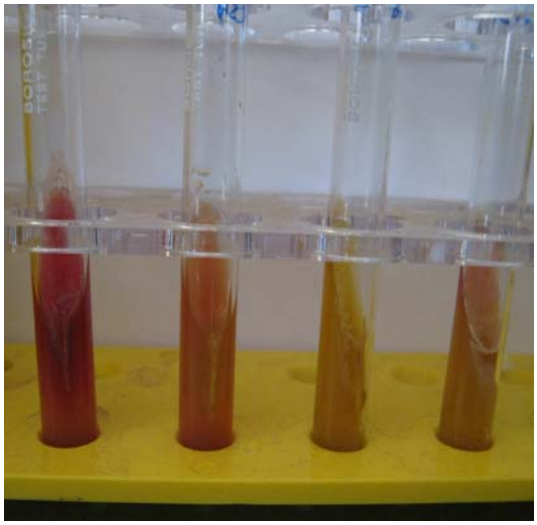
Figure 13: Motility test of isolated bacterial strains in SIM media.

Biochemical identification:

The six bacterial species were biochemically using Himedia rapid biochemical identification kit and were identified to be *Staphylococcus aureus*, *Staphyllococcus salivarius*, *Streptococcus mitis* , *Streptococcus oralis*, *Bacillus subtilis* respectively. All the isolated bacteria were found to be aerobic and most of them were early colonizers of mouth (Fig 14, Fig 15).

Figure 14: Biochemical identification of isolated bacterial strains

Fig 15 (a): Growth in basal media



(b): Triple sugar utilization test

STRESS RESPONSE

SUGAR STRESS:

Biofilm formation was screened using six different sugars as the sole carbon source in basal media and the following results were obtained:

1. Reduction in biofilm intensity was observed when fructose was used as the sole carbon source and G and NW did not show any attachment at all (Table 4, Fig 16.)

Table 4: Elisa reading of biofilm screening in presence of fructose

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.049	0.04	0.048	0.04	0.044	0.04	0.042	0.04	0.041	0.04	0.04
B	0.039	0.050	0.04	0.048	0.039	0.046	0.041	0.047	0.041	0.041	0.04	0.041
C	0.04	0.050	0.039	0.050	0.04	0.046	0.04	0.046	0.041	0.04	0.041	0.04
D	0.04	0.049	0.04	0.048	0.04	0.044	0.04	0.05	0.04	0.041	0.041	0.038
E	0.04	0.041	0.039	0.039	0.039	0.04	0.041	0.04	0.04	0.04	0.04	0.039
F	0.04	0.04	0.04	0.04	0.04	0.038	0.04	0.041	0.04	0.039	0.04	0.04
G	0.041	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.041	0.039	0.04	0.04
H	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04

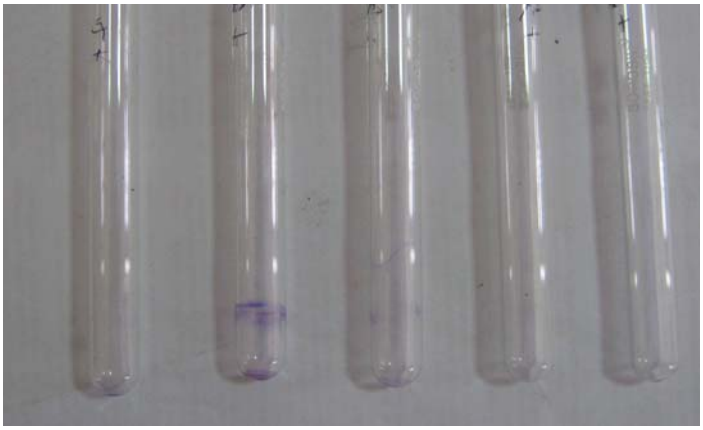


Figure 16: Tube assay for biofilm screening in presence of fructose

2. Intense biofilm formation was observed when sucrose was used as the sole carbon source. (Table 5, Fig 17)

Table 5: ELISA reading of biofilm screening in presence of Sucrose

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.059	0.04	0.059	0.04	0.050	0.04	0.053	0.04	0.059	0.04	0.058
B	0.041	0.059	0.041	0.057	0.041	0.049	0.039	0.054	0.041	0.058	0.039	0.055
C	0.041	0.056	0.041	0.059	0.04	0.053	0.039	0.054	0.04	0.059	0.039	0.058
D	0.04	0.059	0.04	0.062	0.041	0.053	0.041	0.051	0.039	0.058	0.04	0.058
E	0.041	0.04	0.041	0.04	0.04	0.04	0.04	0.04	0.039	0.04	0.041	0.04
F	0.04	0.04	0.041	0.04	0.042	0.04	0.041	0.042	0.039	0.039	0.041	0.04
G	0.04	0.041	0.04	0.041	0.04	0.041	0.04	0.04	0.04	0.039	0.04	0.039
H	0.041	0.04	0.04	0.04	0.04	0.041	0.04	0.04	0.04	0.04	0.04	0.039

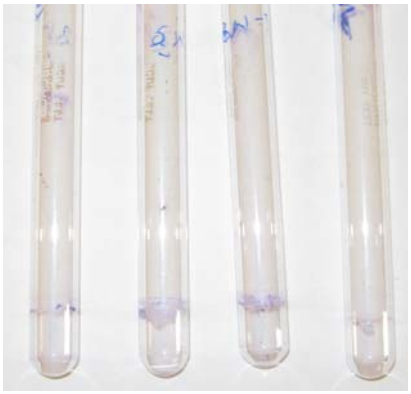


Figure 17: Screening of biofilm formation in presence of sucrose

3. Glucose: Intense biofilm formation was observed when glucose was used as the sole carbon source. (Table 6, Fig 18)

Table 6: ELISA reading of biofilm screening in presence of Glucose

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.055	0.04	0.058	0.04	0.044	0.04	0.05	0.04	0.04	0.04	0.042
B	0.04	0.058	0.04	0.055	0.04	0.044	0.039	0.051	0.041	0.041	0.04	0.04
C	0.041	0.056	0.041	0.058	0.041	0.046	0.039	0.048	0.041	0.041	0.039	0.041
D	0.04	0.055	0.04	0.065	0.041	0.044	0.04	0.05	0.041	0.04	0.039	0.04
E	0.04	0.042	0.04	0.041	0.041	0.04	0.041	0.039	0.04	0.04	0.04	0.039
F	0.042	0.04	0.039	0.04	0.04	0.039	0.041	0.039	0.04	0.041	0.039	0.04
G	0.04	0.039	0.039	0.04	0.04	0.039	0.04	0.04	0.04	0.041	0.04	0.04
H	0.04	0.039	0.04	0.039	0.04	0.04	0.039	0.04	0.04	0.04	0.04	0.041

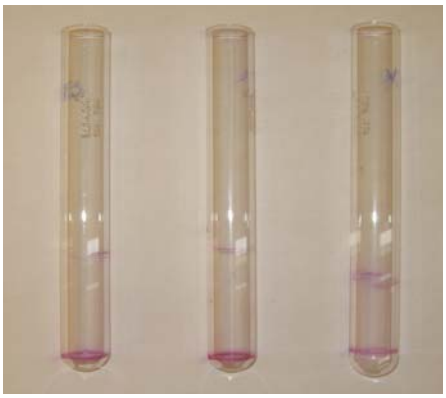


Figure 18: Biofilm screening in presence of Glucose

4. Xylose**:** Intensity of film formation was greatly reduced when xylose was used as the sole sugar source.(Table 7, Fig 19)

Table 7: ELISA reading of biofilm screening in presence of Xylose

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.045	0.04	0.045	0.041	0.044	0.04	0.044	0.04	0.054	0.04	0.057
B	0.041	0.044	0.041	0.045	0.04	0.044	0.04	0.044	0.04	0.049	0.041	0.055
C	0.041	0.043	0.04	0.045	0.04	0.043	0.041	0.045	0.041	0.052	0.04	0.055
D	0.041	0.043	0.04	0.044	0.041	0.04	0.041	0.043	0.04	0.052	0.039	0.049
E	0.041	0.04	0.041	0.04	0.039	0.04	0.04	0.041	0.04	0.042	0.04	0.04
F	0.04	0.041	0.041	0.04	0.04	0.041	0.04	0.04	0.04	0.041	0.04	0.04
G	0.04	0.04	0.041	0.039	0.04	0.04	0.041	0.04	0.041	0.04	0.041	0.041
H	0.041	0.04	0.04	0.04	0.041	0.04	0.04	0.041	0.041	0.04	0.041	0.04

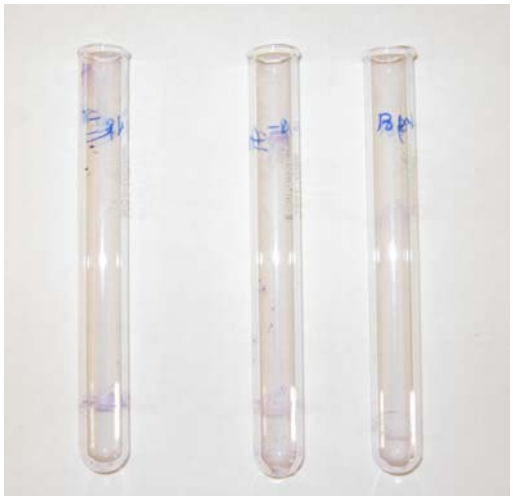


Figure 19: Biofilm screening in presence of Xylose

5. Arabinose: Biofilm formation was negligible when arabinose was used as the sole carbon source.(Table 8)

Table 8: ELISA reading of biofilm screening in presence of Arabinose

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.041	0.042	0.04	0.044	0.04	0.044	0.041	0.044	0.041	0.055	0.041	0.054
B	0.041	0.044	0.04	0.046	0.04	0.046	0.04	0.042	0.041	0.058	0.041	0.057
C	0.04	0.041	0.041	0.049	0.041	0.043	0.041	0.044	0.04	0.054	0.04	0.057
D	0.04	0.042	0.04	0.045	0.04	0.044	0.041	0.044	0.041	0.054	0.04	0.058
E	0.04	0.041	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.041	0.04	0.041
F	0.041	0.04	0.041	0.041	0.04	0.041	0.04	0.041	0.04	0.041	0.041	0.04
G	0.041	0.04	0.041	0.041	0.041	0.041	0.041	0.04	0.041	0.04	0.041	0.041
H	0.041	0.04	0.04	0.042	0.041	0.041	0.041	0.04	0.041	0.041	0.04	0.041

6. Raffinose: Biofilm formation was reduced when raffinose when used as the sole carbone source (Table 9)

Table 9: ELISA reading of biofilm formation in presence of Raffinose

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.042	0.041	0.044	0.04	0.044	0.041	0.046	0.041	0.047	0.04	0.044
B	0.04	0.041	0.041	0.044	0.04	0.045	0.041	0.045	0.041	0.045	0.041	0.045
C	0.041	0.041	0.04	0.045	0.041	0.045	0.04	0.045	0.04	0.045	0.041	0.043
D	0.041	0.042	0.04	0.044	0.04	0.045	0.04	0.046	0.04	0.044	0.041	0.049
E	0.04	0.042	0.04	0.041	0.041	0.04	0.04	0.041	0.041	0.041	0.04	0.041
F	0.04	0.041	0.041	0.041	0.04	0.041	0.041	0.041	0.041	0.04	0.04	0.041
G	0.04	0.041	0.041	0.04	0.04	0.04	0.041	0.041	0.041	0.04	0.041	0.04
H	0.041	0.04	0.04	0.041	0.041	0.04	0.041	0.04	0.04	0.04	0.041	0.04

Salt stress: The intensity of biofilm formation reduced gradually with increase in salt concentration.(Table 10, 11, 12)

Table10: Absorbance at salt conc. 0.6 mg/ml

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.057	0.04	0.05	0.04	0.049	0.04	0.047	0.04	0.056	0.04	0.056
B	0.04	0.057	0.041	0.048	0.041	0.046	0.04	0.048	0.041	0.052	0.041	0.056
C	0.041	0.058	0.041	0.052	0.041	0.046	0.041	0.046	0.041	0.055	0.041	0.058
D	0.041	0.055	0.041	0.049	0.042	0.058	0.041	0.046	0.04	0.052	0.04	0.056
E	0.04	0.04	0.039	0.041	0.04	0.041	0.041	0.042	0.04	0.041	0.04	0.04
F	0.04	0.041	0.04	0.041	0.04	0.041	0.04	0.04	0.041	0.04	0.04	0.041
G	0.039	0.04	0.04	0.04	0.04	0.041	0.04	0.04	0.041	0.041	0.041	0.04
H	0.04	0.04	0.04	0.041	0.041	0.04	0.04	0.041	0.04	0.04	0.041	0.04

Table 11: Absorbance in presence of 0.8mg/ml of salt concentration

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.049	0.04	0.048	0.041	0.044	0.04	0.051	0.041	0.051	0.04	0.057
B	0.041	0.051	0.041	0.049	0.04	0.043	0.041	0.051	0.04	0.051	0.04	0.057
C	0.04	0.051	0.04	0.047	0.04	0.045	0.041	0.049	0.04	0.053	0.041	0.059
D	0.04	0.052	0.04	0.05	0.039	0.042	0.04	0.051	0.041	0.052	0.041	0.058
E	0.041	0.042	0.041	0.041	0.041	0.041	0.04	0.041	0.041	0.04	0.04	0.04
F	0.041	0.041	0.04	0.041	0.04	0.04	0.041	0.04	0.04	0.04	0.04	0.041
G	0.04	0.04	0.041	0.04	0.041	0.041	0.04	0.041	0.04	0.041	0.041	0.04
H	0.04	0.04	0.04	0.041	0.041	0.04	0.04	0.041	0.04	0.04	0.04	0.04

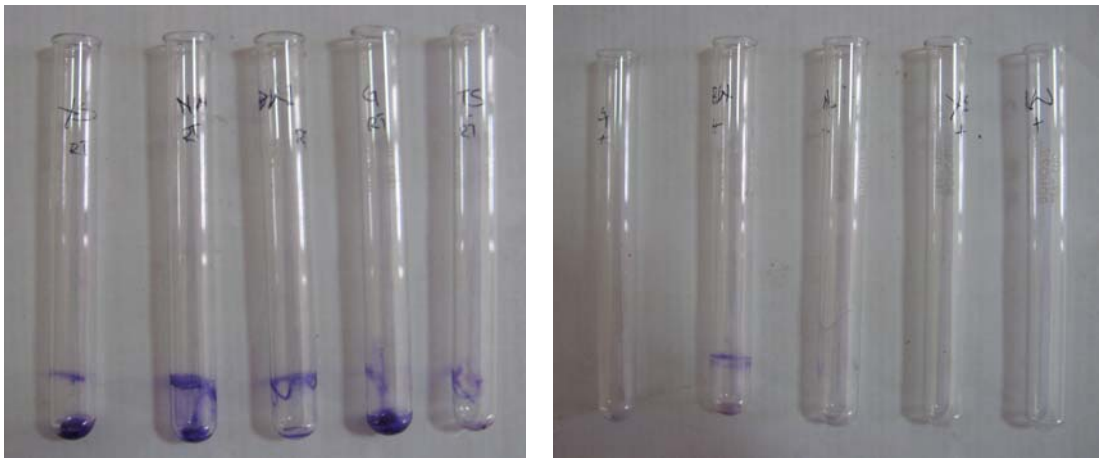
Table 12: Absorbance in presence of 1mg/ml of salt concentration

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.042	0.041	0.044	0.04	0.044	0.04	0.049	0.04	0.052	0.041	0.053
B	0.041	0.043	0.04	0.045	0.041	0.044	0.041	0.051	0.04	0.051	0.04	0.051
C	0.041	0.043	0.041	0.043	0.041	0.043	0.041	0.05	0.042	0.051	0.04	0.052
D	0.042	0.043	0.041	0.043	0.041	0.045	0.042	0.05	0.04	0.05	0.04	0.052
E	0.04	0.04	0.04	0.04	0.04	0.041	0.04	0.04	0.041	0.041	0.041	0.04
F	0.04	0.04	0.041	0.041	0.041	0.041	0.04	0.041	0.041	0.04	0.04	0.041
G	0.041	0.041	0.041	0.041	0.041	0.04	0.041	0.041	0.041	0.04	0.041	0.04
H	0.04	0.04	0.04	0.04	0.04	0.04	0.041	0.04	0.04	0.04	0.041	0.04

Temperature stress: The intensity of biofilm formation did not vary much with change in temperature (Table 13-15, Fig 20)

Table 13: Absorbance at room temperature

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.058	0.041	0.05	0.041	0.049	0.04	0.051	0.04	0.057	0.041	0.062
B	0.04	0.058	0.04	0.053	0.04	0.047	0.041	0.051	0.04	0.057	0.041	0.062
C	0.041	0.058	0.041	0.053	0.04	0.049	0.041	0.051	0.041	0.062	0.04	0.063
D	0.041	0.055	0.041	0.053	0.041	0.049	0.041	0.05	0.041	0.055	0.04	0.062
E	0.04	0.04	0.041	0.04	0.04	0.04	0.04	0.04	0.041	0.041	0.04	0.041
F	0.041	0.04	0.04	0.041	0.04	0.041	0.04	0.04	0.04	0.041	0.041	0.04
G	0.041	0.041	0.04	0.04	0.041	0.041	0.04	0.04	0.04	0.041	0.041	0.04
H	0.041	0.04	0.041	0.04	0.041	0.04	0.041	0.041	0.04	0.04	0.041	0.04



(a) **(b)**

Figure 20: Screening for biofilm formation (a)At room temp.(b)At 40ºC

Table 14: Absorbance at 37ºC

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.06	0.041	0.049	0.04	0.047	0.041	0.05	0.04	0.05	0.041	0.053
B	0.041	0.059	0.04	0.05	0.04	0.049	0.04	0.049	0.041	0.049	0.04	0.051
C	0.041	0.061	0.04	0.051	0.041	0.049	0.041	0.049	0.04	0.052	0.041	0.053
D	0.04	0.062	0.041	0.049	0.04	0.047	0.039	0.048	0.04	0.052	0.04	0.053
E	0.039	0.041	0.04	0.04	0.041	0.04	0.039	0.041	0.039	0.041	0.04	0.041
F	0.04	0.04	0.041	0.04	0.041	0.041	0.04	0.04	0.041	0.04	0.041	0.041
G	0.039	0.04	0.041	0.041	0.041	0.041	0.041	0.041	0.04	0.04	0.04	0.04
H	0.04	0.041	0.041	0.04	0.04	0.04	0.04	0.041	0.04	0.041	0.04	0.041

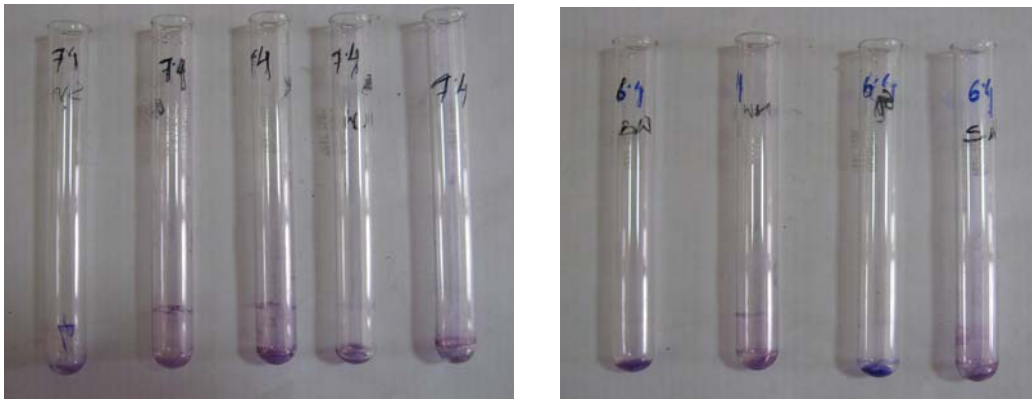
Table 15: Absorbance at 26ºC

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.06	0.041	0.049	0.04	0.047	0.041	0.05	0.04	0.05	0.041	0.053
B	0.041	0.059	0.04	0.05	0.04	0.049	0.04	0.049	0.041	0.049	0.04	0.051
C	0.041	0.061	0.04	0.051	0.041	0.049	0.041	0.049	0.04	0.052	0.041	0.053
D	0.04	0.062	0.041	0.049	0.04	0.047	0.039	0.048	0.04	0.052	0.04	0.053
E	0.039	0.041	0.04	0.04	0.041	0.04	0.039	0.041	0.039	0.041	0.04	0.041
F	0.04	0.04	0.041	0.04	0.041	0.041	0.04	0.04	0.041	0.04	0.041	0.041
G	0.039	0.04	0.041	0.041	0.041	0.041	0.041	0.041	0.04	0.04	0.04	0.04
H	0.04	0.041	0.041	0.04	0.04	0.04	0.04	0.041	0.04	0.041	0.04	0.041

pH Stress: The intensity of biofilm formation was gradually increased with a decrease in pH value.(Table 16-19,Fig 21, 22)

Table 16: Absorbance at pH 7.4

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.062	0.041	0.049	0.041	0.046	0.041	0.052	0.04	0.055	0.04	0.057
B	0.041	0.057	0.04	0.052	0.04	0.046	0.041	0.051	0.041	0.052	0.041	0.055
C	0.04	0.056	0.04	0.051	0.04	0.049	0.04	0.049	0.039	0.053	0.04	0.055
D	0.04	0.056	0.041	0.049	0.041	0.052	0.04	0.049	0.04	0.053	0.041	0.056
E	0.039	0.042	0.04	0.04	0.039	0.041	0.041	0.041	0.039	0.042	0.04	0.041
F	0.04	0.041	0.041	0.041	0.04	0.04	0.041	0.041	0.041	0.04	0.04	0.04
G	0.04	0.041	0.04	0.041	0.04	0.04	0.04	0.04	0.041	0.041	0.041	0.041
H	0.04	0.04	0.04	0.041	0.041	0.04	0.041	0.04	0.04	0.041	0.04	0.041



(a) **(b)**

Figure 21: Screening for biofilm formation (a) At pH 7.4 (b) At pH 8.4

Table 17: Absorbance at pH 6.4

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.039	0.052	0.04	0.048	0.04	0.043	0.04	0.047	0.04	0.049	0.041	0.049
B	0.04	0.052	0.041	0.049	0.041	0.049	0.04	0.048	0.041	0.049	0.04	0.048
C	0.04	0.053	0.04	0.05	0.04	0.049	0.041	0.047	0.04	0.048	0.039	0.049
D	0.039	0.052	0.041	0.049	0.04	0.044	0.039	0.049	0.039	0.047	0.041	0.049
E	0.041	0.04	0.041	0.039	0.039	0.04	0.04	0.04	0.041	0.041	0.04	0.041
F	0.041	0.041	0.04	0.041	0.04	0.041	0.039	0.039	0.04	0.041	0.04	0.039
G	0.04	0.04	0.04	0.04	0.041	0.041	0.041	0.04	0.04	0.04	0.041	0.04
H	0.041	0.04	0.04	0.041	0.04	0.04	0.04	0.041	0.04	0.041	0.04	0.041

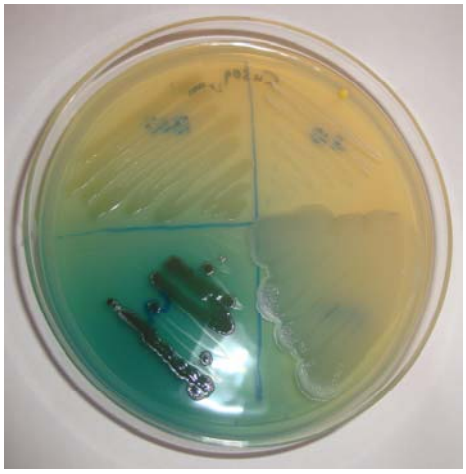
Table 18: Absorbance at pH 5.4

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.048	0.04	0.043	0.041	0.044	0.04	0.043	0.041	0.045	0.04	0.047
B	0.039	0.049	0.041	0.046	0.041	0.043	0.041	0.044	0.04	0.044	0.041	0.046
C	0.041	0.048	0.04	0.046	0.039	0.044	0.04	0.043	0.04	0.044	0.04	0.046
D	0.041	0.048	0.039	0.047	0.041	0.044	0.041	0.046	0.041	0.044	0.04	0.046
E	0.039	0.04	0.041	0.04	0.04	0.039	0.04	0.041	0.04	0.041	0.039	0.04
F	0.04	0.041	0.04	0.039	0.039	0.041	0.04	0.04	0.041	0.04	0.04	0.041
G	0.041	0.04	0.041	0.04	0.04	0.04	0.041	0.04	0.04	0.04	0.04	0.039
H	0.04	0.041	0.04	0.041	0.041	0.041	0.04	0.041	0.041	0.041	0.041	0.04

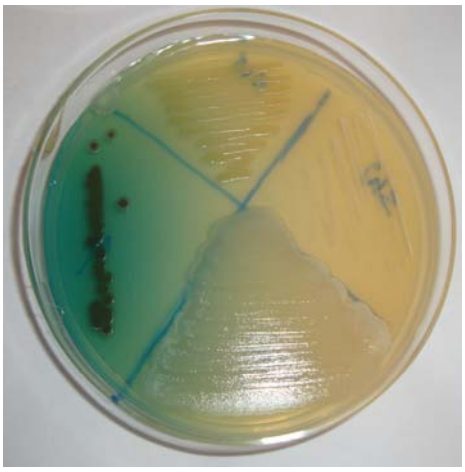
Figure 22: Biofilm screening at pH 8.4

Metal Stress:

Moderate biofilm formation was observed at 10 ppm of Zn^{2+} but at higher concentrations biofilm formation was completely inhibited.(Fig 23 a-d,24)



(a)



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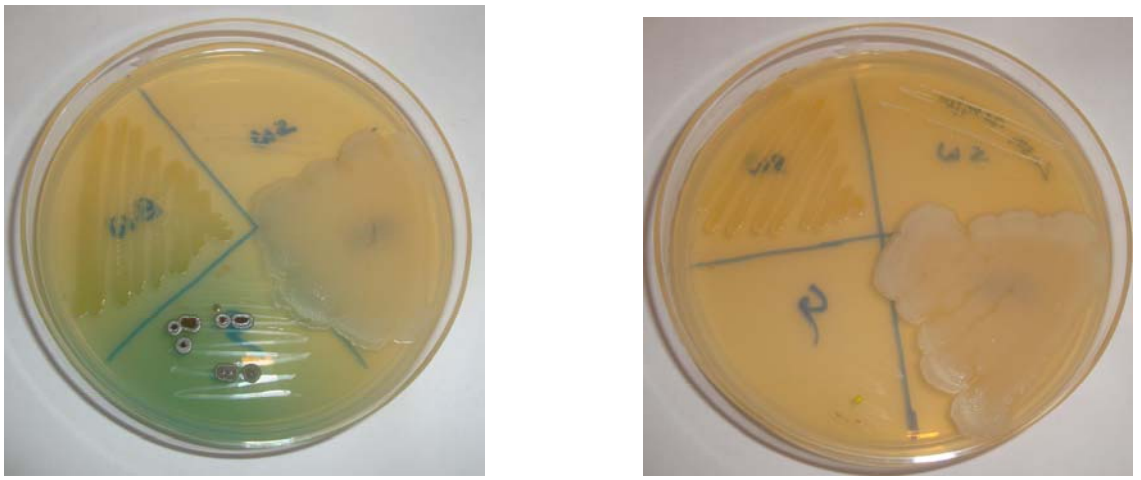


(b) **(d)**

Figure 23: Plates shoing growth patterns of the biofilm forming strains under study at (a) 10 ppm, (b) 20 ppm, (c) 40 ppm, (d) 80 ppm of Zn^{2+} concentration

Figure 24: Tubes showing biofilm attachment in presence of 10 ppm concentration of Ca^{2+}

Profuse biofilm formation was observed at low to high concentrations of Ca^{2+} as well as Cu^{2+} ions. Decreased film formation was observed at Cd^{2+} and Fe^{2+} ion concentration above 40 ppm. Zinc (Zn^{2+}) inhibited biofilm formation even at concentrations as low as 10ppm.

Antibiotic stress:

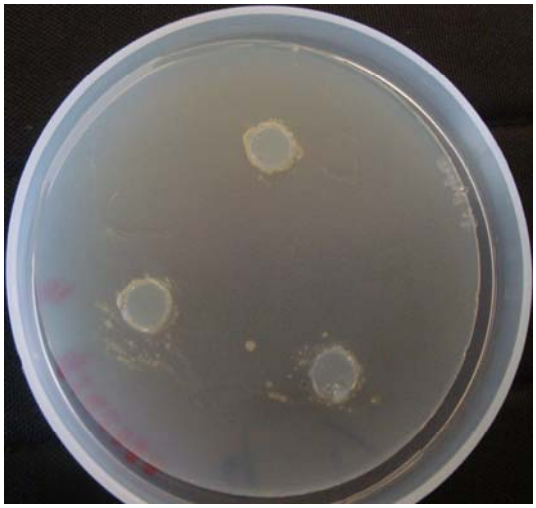
No inhibition of biofilm formation was observed in presence of any antibiotic studied.

Chemotaxis:

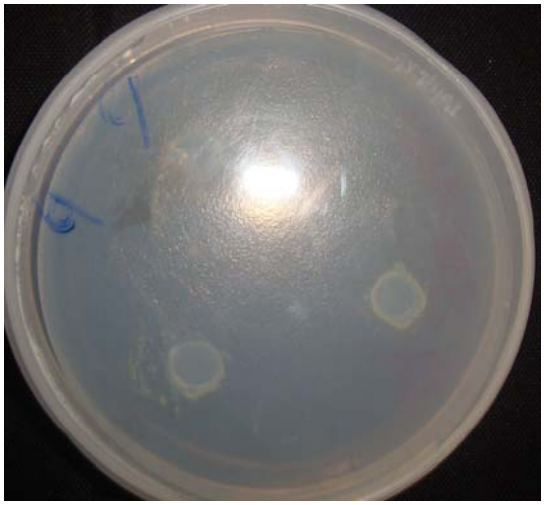
Chemotactic response of 4 strains (BW, SW, G, NW) towards sugars (Fructose, Sucrose, Arabinose) and salts ($FeSO_4$, $PbCl_2$, $CaCl_2$) were studied and showed following results.It was observed that G and NW showed negative response towards fructose and sucrose and BW and SW showed negative results for arabinose while in case of metals showed chemotactic movement towards $CaCl_2$ where as none of the strains showed positive response towards $PbCl_2$ (Table 19, Fig 25)

Table 19: Chemotactic response

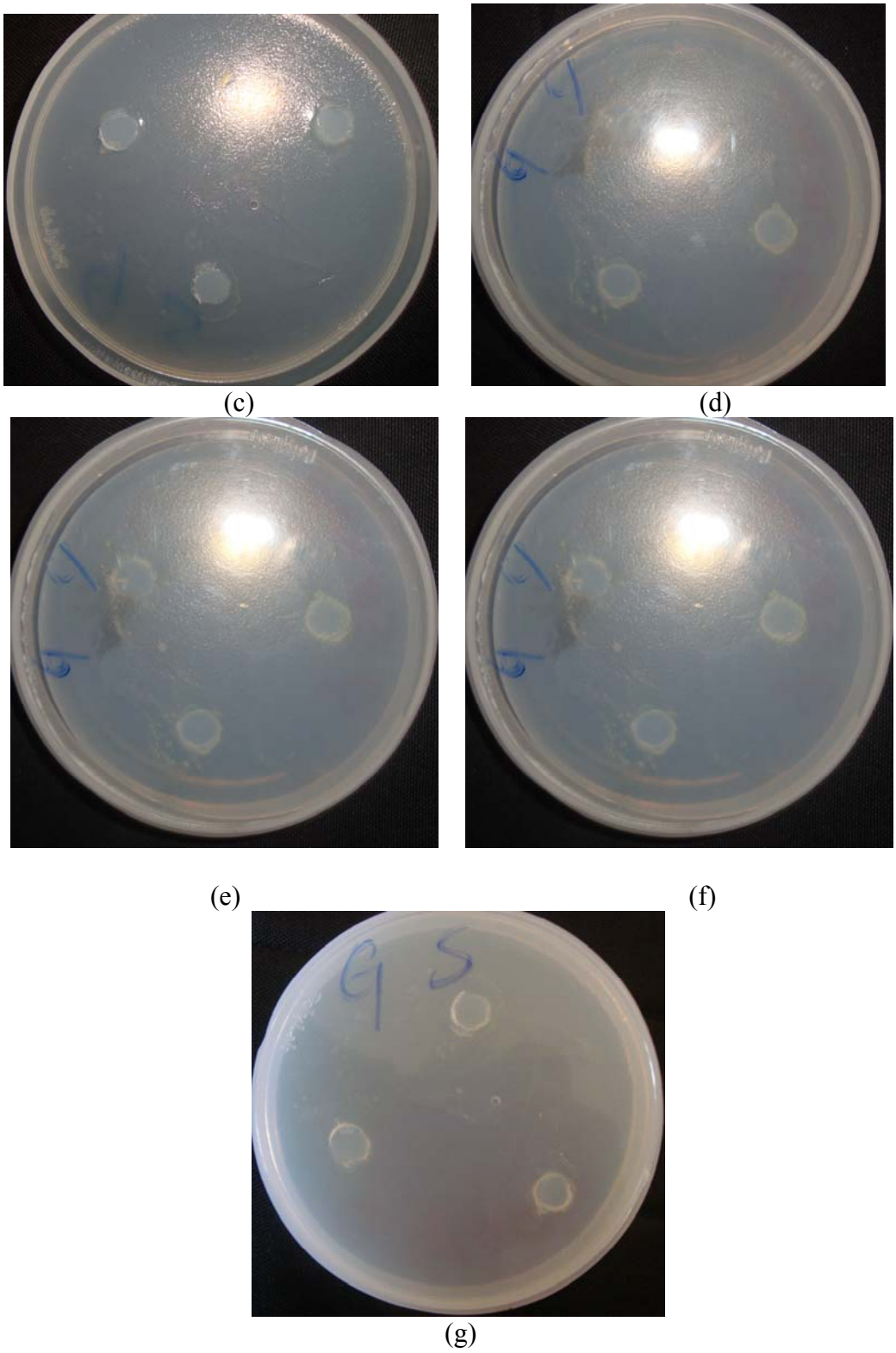
Bacterial strains	Fructose	Sucrose	Arabinose	$FeSO_4$	$PbCl_2$	$CaCl_2$
BW	+	+	-	+	-	+
SW	+	+	-	+	-	+
G	-	-	+	-	-	+
NW	-	-	+	+	-	+



(a)



(b)



(c) (d)

(e) (f)

(g)

Figure 25 : Plates showing chemotactic response of a) BW to $FeSO_4$, b) G to $CaCl_2$, c) BW to Sucrose, d) SW to sucrose, e) NW to Arabinose, f) NW to $FeSO_4$, g) BW to $PbCl_2$ (Negative).

PLANKTONIC GROWTH KINETICS

The growth kinetics of isolated strains in planktonic state was observed and the following trend were observed (Figs 26-31)

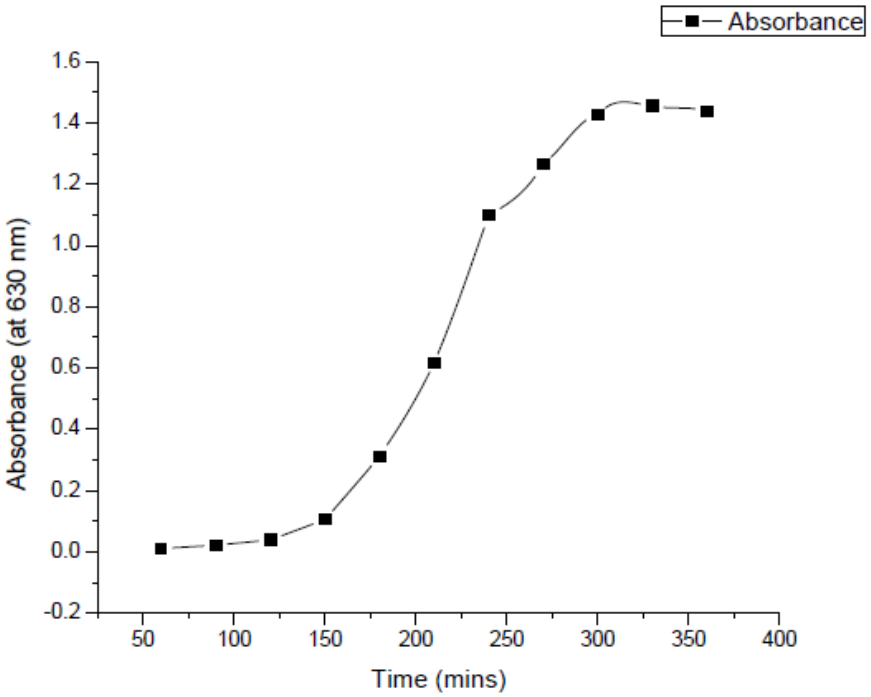


Figure26. Planktonic growth curve of YS

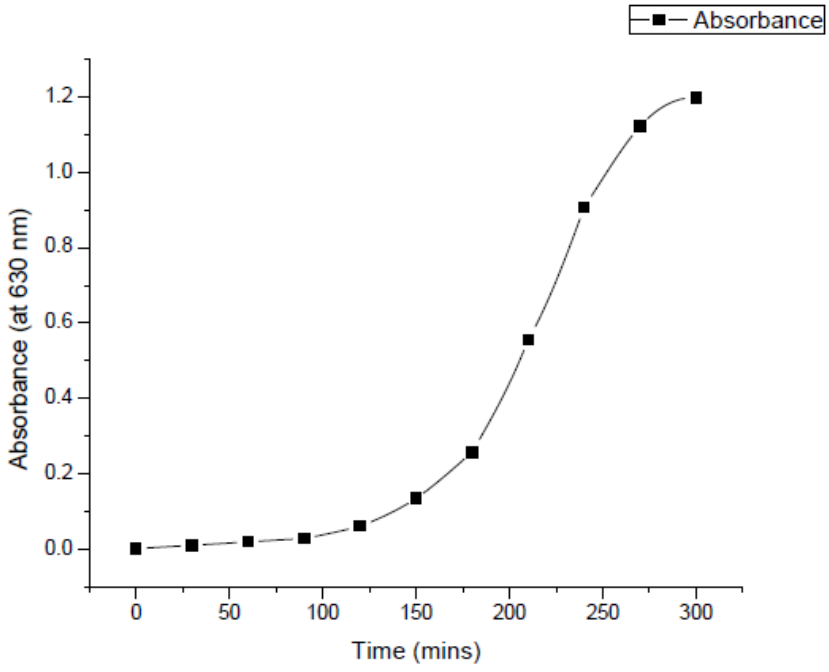


Figure 27. Planktonic growth curve of TS

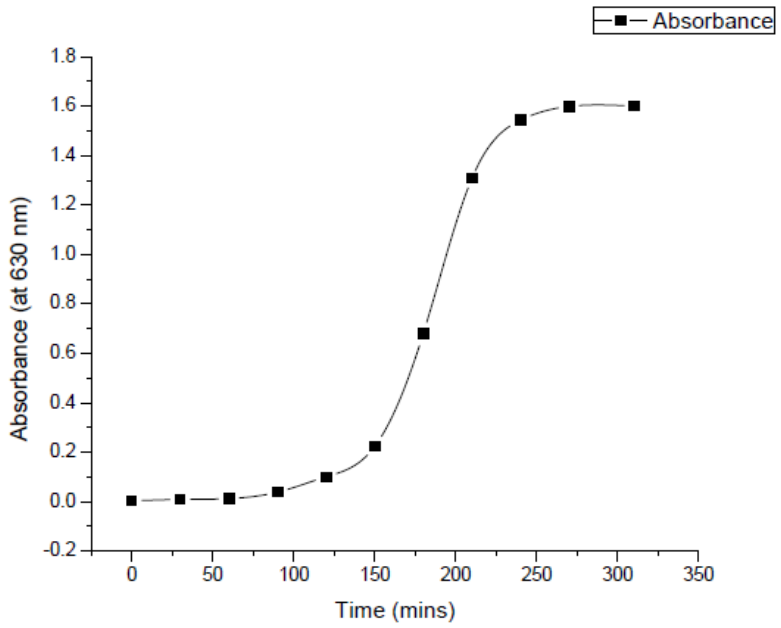


Figure28. Planktonic growth curve of SW

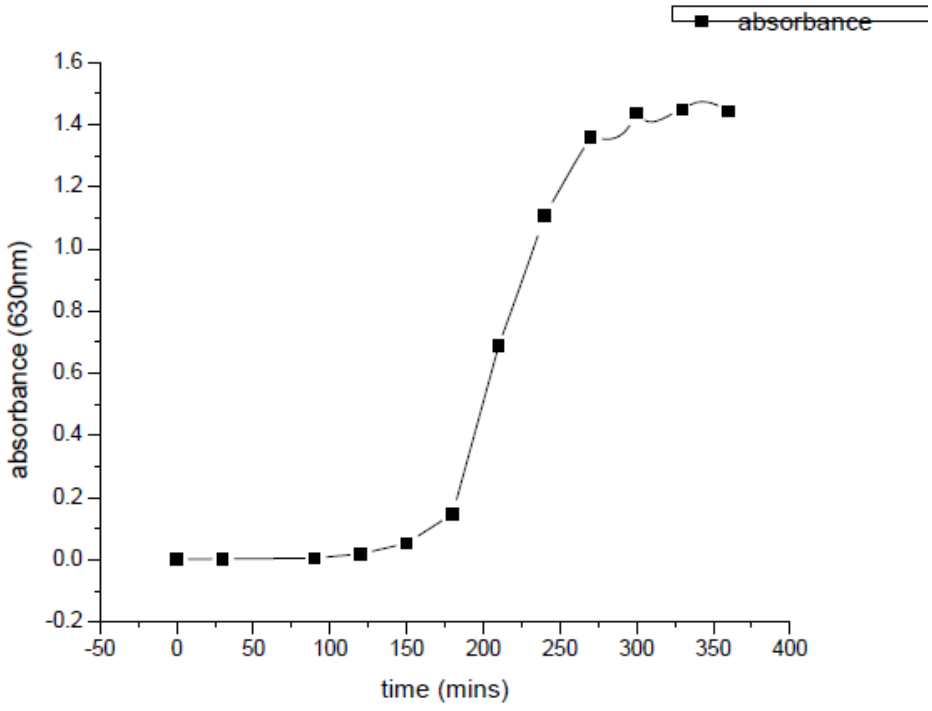


Figure29. Planktonic growth curve of NW

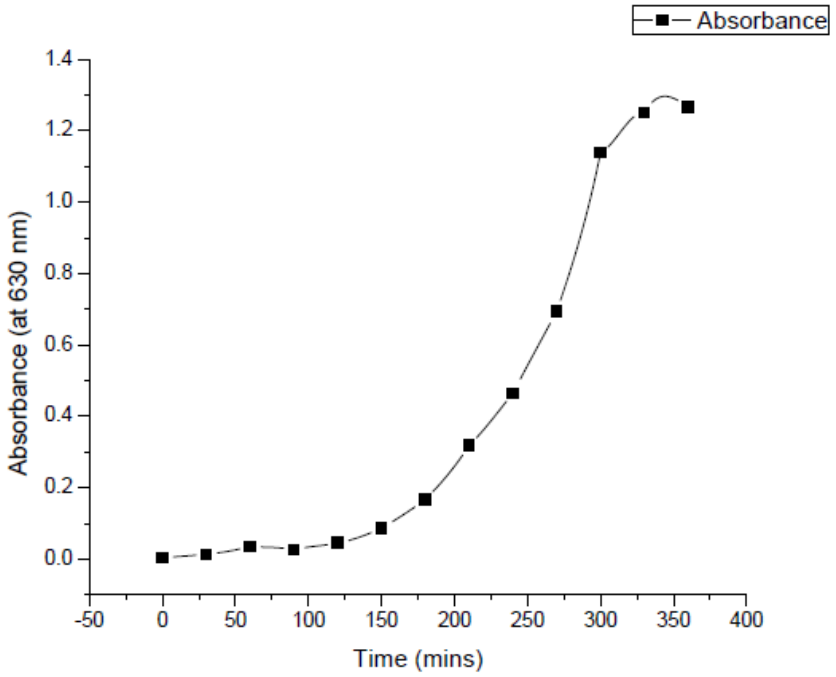


Figure30. Planktonic growth curve of G

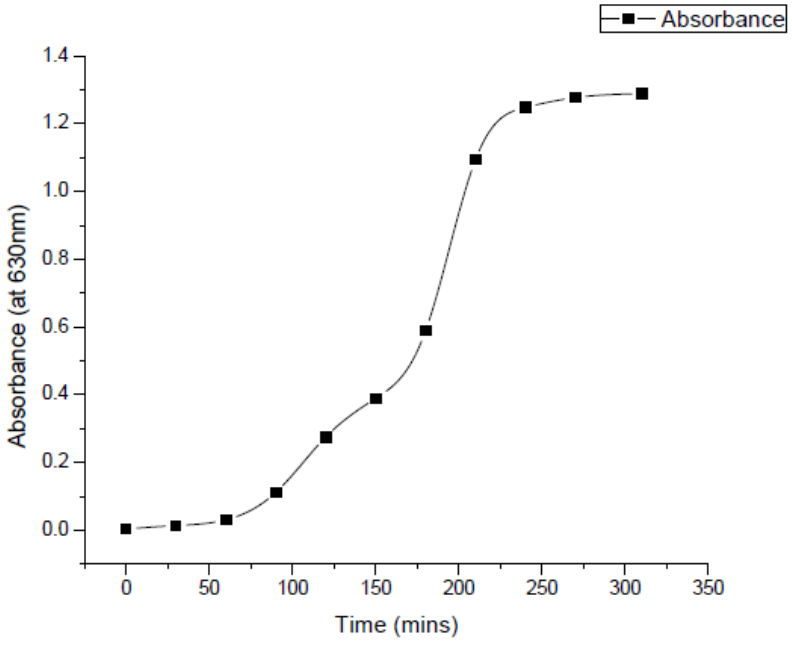


Figure31. Planktonic growth curve of BW

Kinetics of biofilm formation:

The kinetics of biofilm formation was studied and the following trend was observed. All strains showed decline after 7 days due to disintegration where as NW showed disintegration after 10 days and BW after 5 days (Fig 32-37).

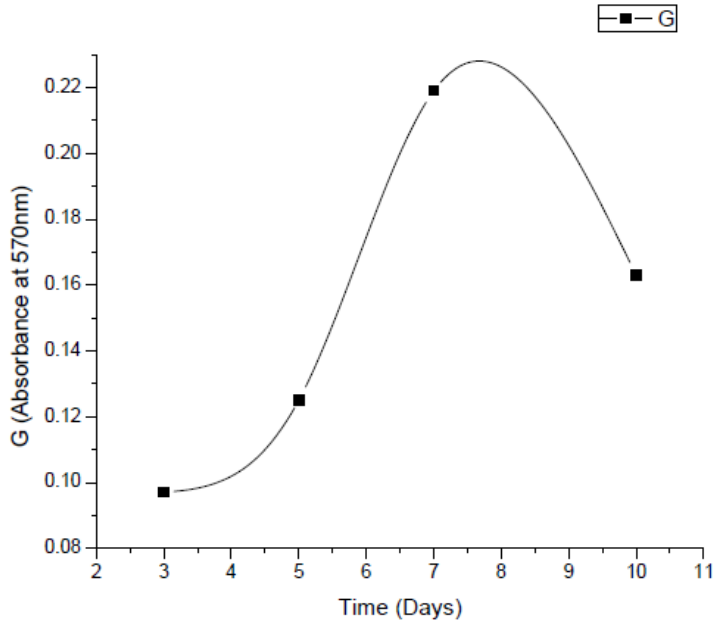


Figure32. Biofilm formation kinetics of G

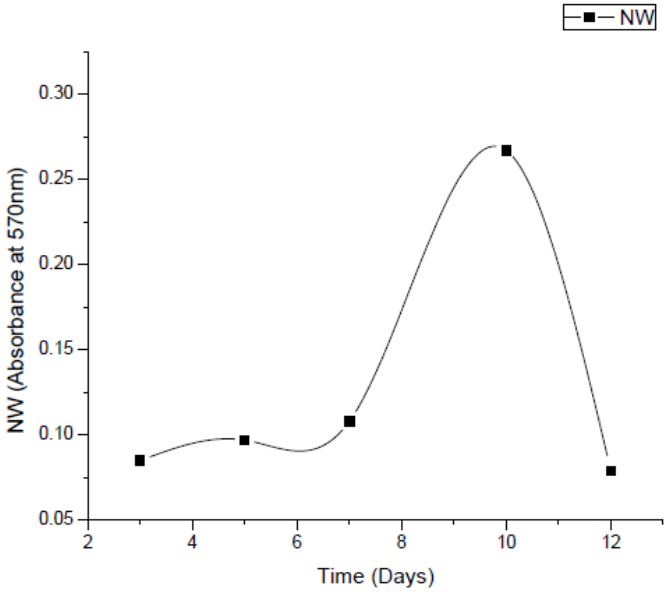


Figure 33. Biofilm formation kinetics of NW

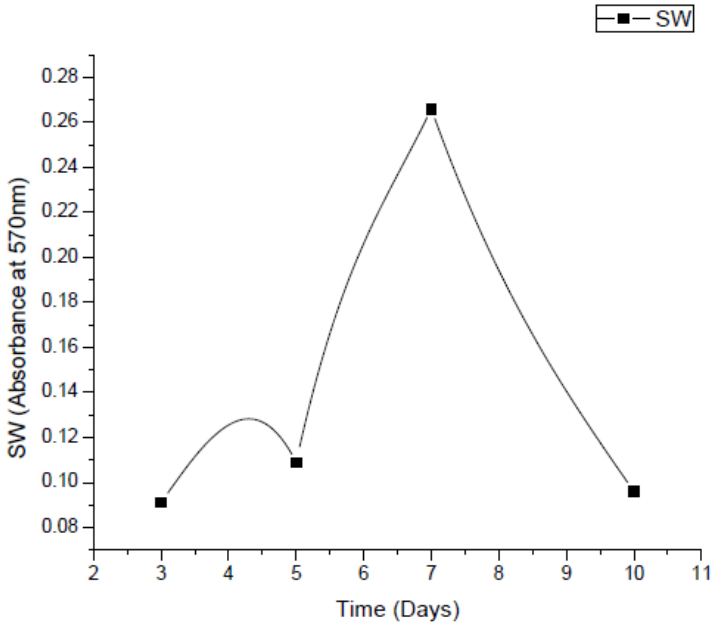


Figure 34. Biofilm formation kinetics of SW

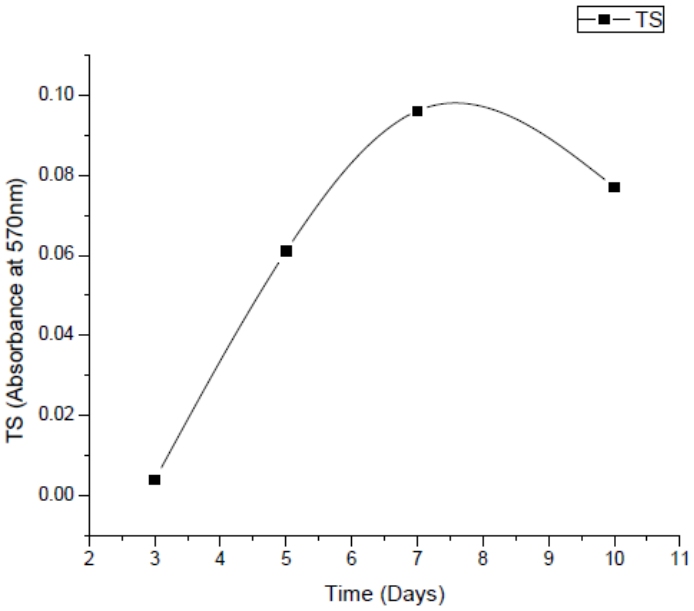


Figure 35. Biofilm formation kinetics of TS

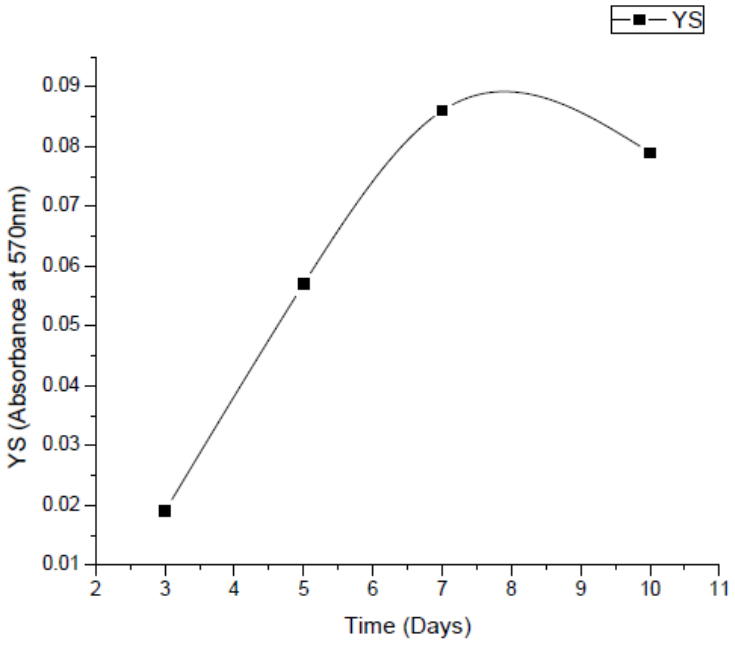


Figure: 36 Biofilm formation in YS

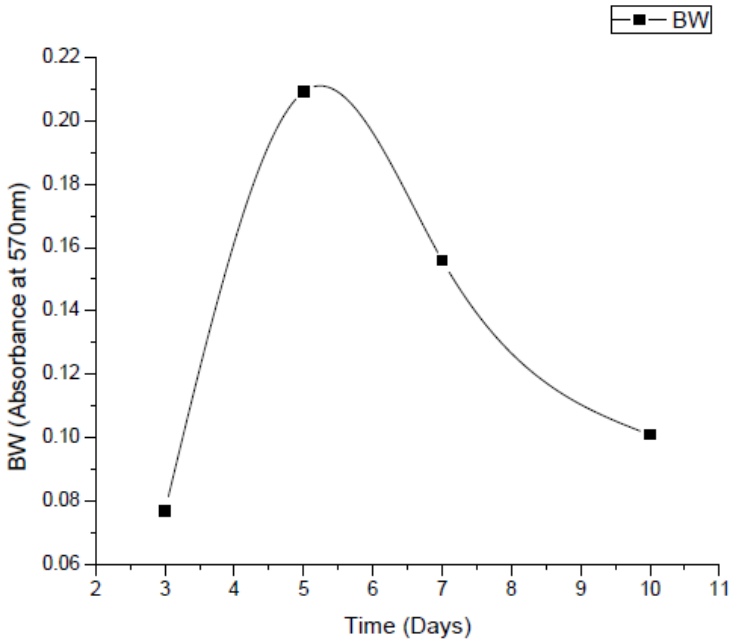


Figure37. Biofilm formation in BW

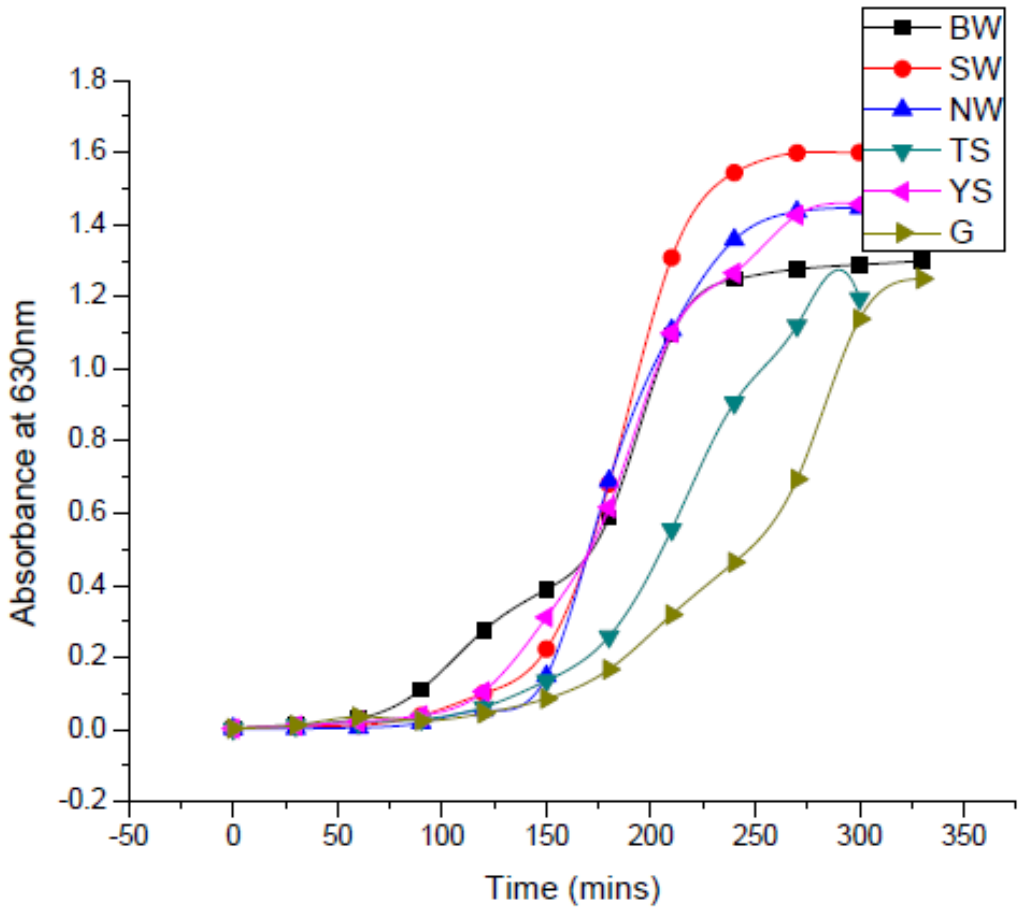


Figure 38. Comparative analysis of planktonic growth curve of BW, SW, NW, TS, YS, G showed that all isolated strains showed general trend except G which is slow growing.

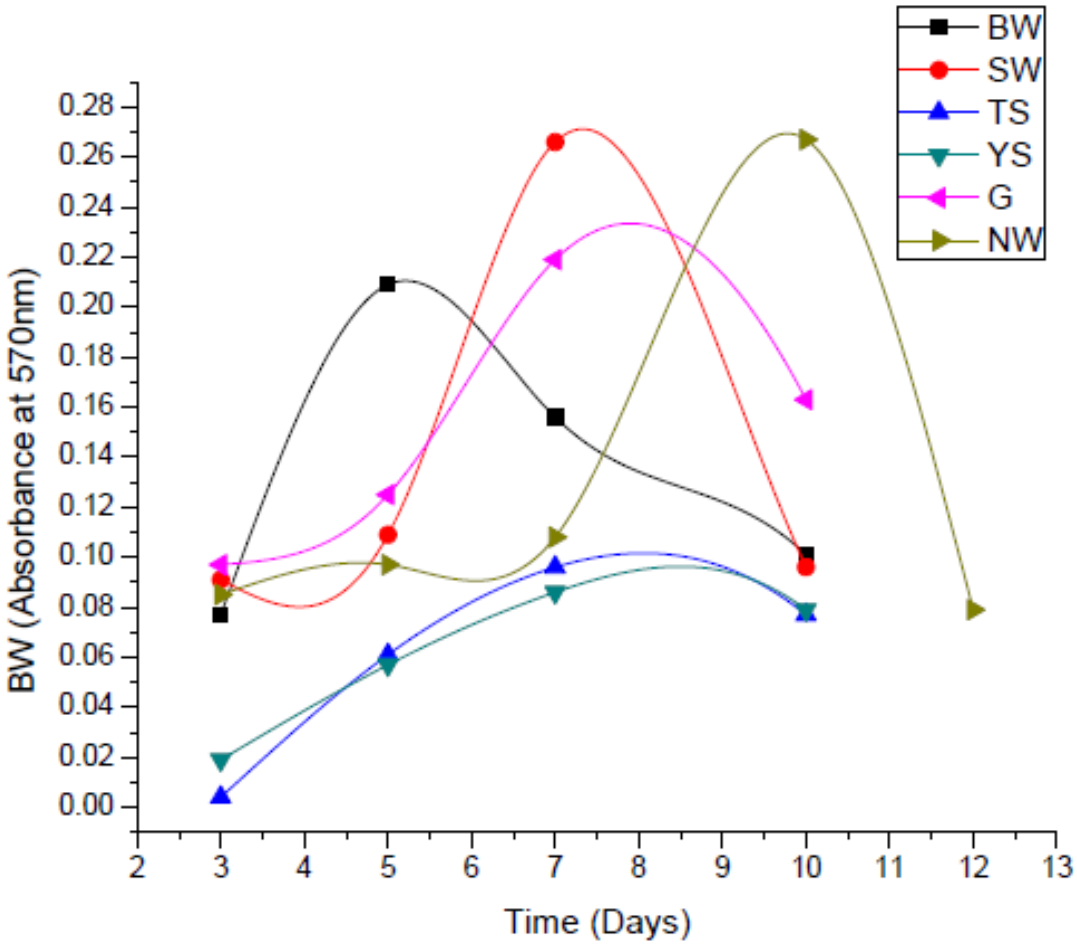


Figure39. Comparative analysis of biofilm formation kinetics of BW, SW, TS, YS, G and NW showed that NW secreted EPS for maximum days(10) where as all other disintegrated after 7 days except BW which started disintegrating after 5 days (Fig 33-38)

Characterization of EPS Matrix:

The extracted EPS was analysed using FTIR spectroscopy for study of its components. The graphs obtained for the strains BW, SW, G and NW are given in Fig. 40-43. The presence of –CH—vibrations in lipids, amide I in proteins, amide II in protein and –COC—group vibration in carbohydrates, DNA and RNA is indicated by the peaks obtained at wave no. range 3200-2800, 1800-1600, 1600-1500 and 1200-800 cm^{-1} resp. obtained from the FTIR analysis of the EPS matrix. The results of chemical analysis are given in Table 20.

FTIR analysis

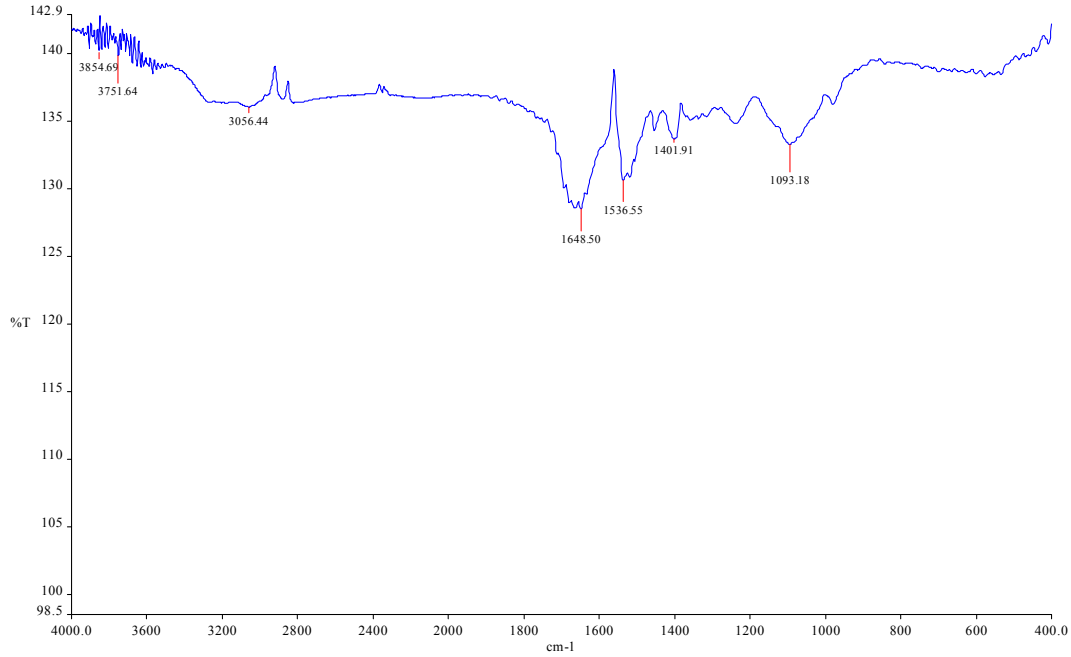


Figure 40 : FTIR analysis of EPS matrix formed by G

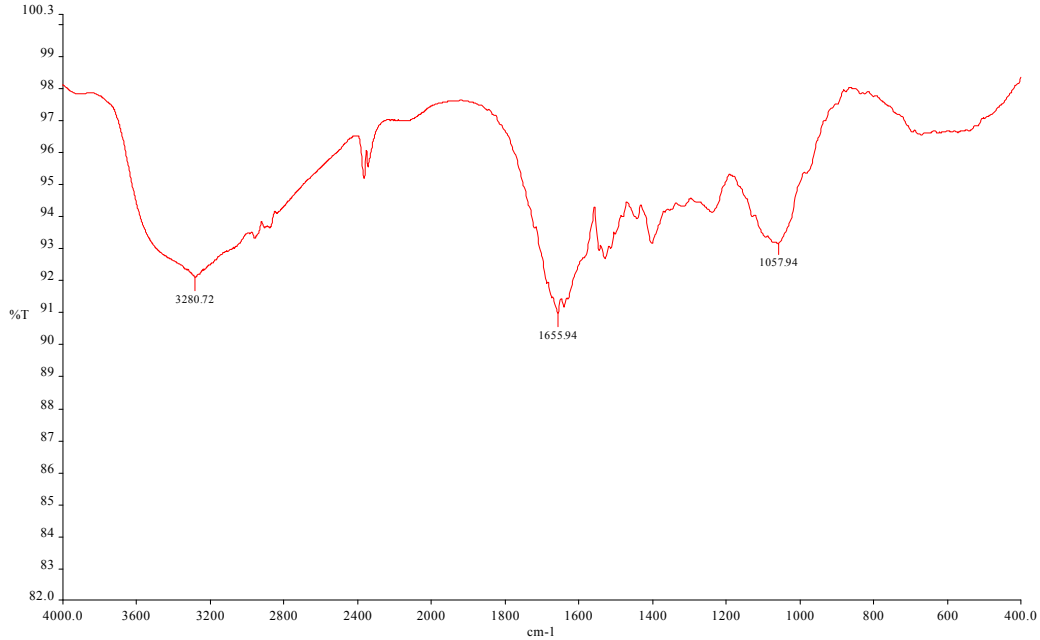


Figure 41:FTIR analysis of EPS matrix formed by NW

Figure 42: FTIR analysis of EPS matrix produced by BW

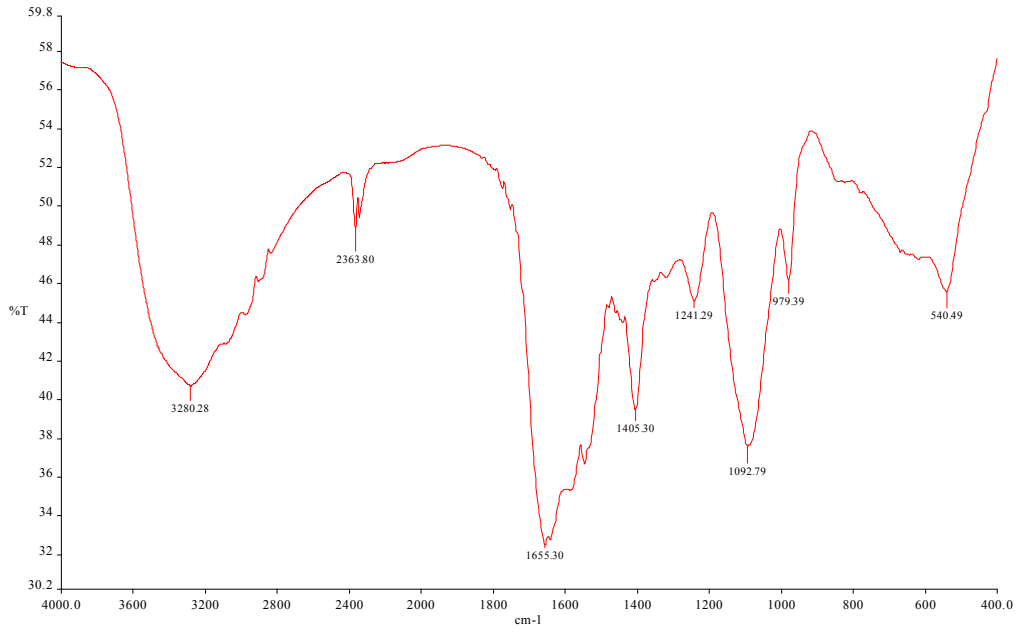


Figure 43: FTIR analysis of EPS matrix produced by SW

Table 20: Chemical analysis of EPS matrix:

Bacterial strains	Protein content in µg/ml	Carbohydrate content in µg/ml	DNA content in µg/ml
BW	**1467**	**148.8**	**2.39**
SW	**1248**	**117.9**	**1.19**
G	**1367.5**	**154.8**	**5.11**
NW	**1698**	**105.9**	**2.48**

Microbiological studies reveal that the microbial flora associated with chronic gingivitis and periodontitis is extremely complex aggregation of over 700 species of bacteria. (Moore et al., 1982, 1983). Periodontis and gingivitis are mediated by toxic substances released by dental plaque bacteria. These toxins are cytotoxic growth inhibiting substances produced from matured biofilm as a result of complex association of bacteria whose properties get modified due to genetic changes within the biofilm.(Levine, 1985) These toxins inhibit the growth of beneficial bacteria thereby disturbing the microbial ecology of the entire body.

Cariogenity results due to fermentation of carbohydrates by the plaque bacteria producing acidic environment conducive for growth of cariogenic bacteria further intensifying the film. In the present study, biofilm formation was checked under different stress conditions including different sugar source. The maximum filming was observed in presence of Sucrose and Glucose. Carbohydrates, especially sucrose is fermented by the cariogenic bacteria to Glucans, Levans and acids. Glucans and Levans promote formation of dense and more resistant plaque while the acid produced causes demineralization of teeth.

Test for biofilm formation in presence of metal salts of Iron and Zinc showed moderate ho high inhibition o film formation. These results are in agreement with earlier studies conducted where in children in lower scoieconomic groups were fed on supplemented with Zn salts and a reduction in plaque formation was observed. (dieYasemin et al, 2010). . Both ferric- and ferrous-sucrose meals reduced the incidence of smooth-surface and sulcal caries in rats. Stain formation, organic acid production and acidogenic activity of dental plaque in animals receiving iron-sucrose meals were also investigated. Ferric glycerophosphate did not induce extrinsic staining on the tooth surface. The concentration of organic acids did not differ significantly among the groups. The acidogenic activity of plaque in animals receiving either of the iron-sucrose meals tended to be lower. (Miquel et al, 1997).In our present study however, the inhibition of film formation in presence of Iron salts was moderate in case of some strins while some other strains did not form films at all.

These bacteria show an interesting pattern of utilization of different energy sources influencing the pattern of film formation. The maximum film formation was observed in presence of glucose and sucrose but there was a steep decrease in film formation in presence of some very common sugars like fructose. Almost no filming was observed in presence of less common sugars like Xylose, Arabinose and Raffinose. Inhibition was also observed in salts of Cadmium and lead but these elements are known to be hazardous to body even in minute quantitien , hence cannot be used as a preventive measure.

The results obtained from the current from the current studies suggest that , although dental plaque biofilms are tough to control and eradicate, some preventive measure can be taken into consideration.

Chewing gum is a unique food because it is chewed for a prolonged period , while at the same time it contributes relatively few calories.

When chewed by healthy subjects, the flow of saliva increases from a resting value of 0.4-0.5ml/min, to approximately 5-6ml/min, falling after about 5min to around 2ml/min, and slowly thereafter to 1.2-1.5 at 20ml/min.The effect of stimulation is to increase the concentration of bicarbonate from the saliva entering the mouth. This bicarbonate raises the pH of the saliva, and greatly increases its buffering power; the saliva is therefore much more effective from neutralizing and buffering food acids and acids arising from plaque from the fermentation of carbohydrate. At the same time, the phosphate of saliva changes as a result of the rise from pH, so that a higher proportion of it is from the form of PO4-.These changes from the composition of stimulated saliva lead to a greater ability to prevent a fall from pH, and a greater tendency to favour hydroxyapatite crystal growth. In addition, the greater volume and rate of flow of stimulated saliva results from an increased ability to clear sugars and acids from around the teeth thereby reducing plaque accumulation. This can be used as the most effective strategy in preventing biofilm formation on tooth surface. As such chewing causes mechanical diaggregation by virtue of the frictional force it exerts on the teeth surface. Incorporation of effective Antibiotics, Salts of various metals and other compounds showing inhibitory effect some natural compounds in these chewing gums can prove to be really effective in battling against these biofilm formers. These gums can also be made to maintain a particular pH with suitable alteration in chemical composition so that it inhibits the survival and growth of the opportunistic pathogens.

Use of mouthwashes is often prescribed for general oral health. Incorporation of moderate amounts of metal salts known to prevent filming along with some effective antibiotics can be helpful in coating the teeth enamel with these compounds rendering it less susceptible to colonization. These mouthwashes can be designed as suspension of micro to nanoparticles of these effective compounds for better efficiency.

The project undertaken herein is only a preliminary work based on classical approach. However, intensive research is urged by this alarming problem of dental plaque biofilm which not only affects oral health but also puts the general human health at a risk.

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