STUDIES ON ISOLATION AND CHARACTERIZATION OF ORAL BIOFILM 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 and characterization of oral biofilm forming bacteria. The major constituent of biofilm other than bacterial cells is the Extracellular Polymeric Slime matrix (EPS) which is secreted by the bacterial cells themselves. Physical properties of biofilms like attachment, mechanical strength, antibiotic resistance can be attributed to EPS matrix. In this study, attempts were made to study the stress response of bacteria isolated and their chemotactic response. Further attempts were made to characterize the EPS matrix by chemical as well as spectroscopic studies. The bacterial strains isolated were characterized to be *Staphylococcus aureus*, *Staphylococcus salivarius*, *Streptococcus mitis*, *Streptococcus oralis*, *Bacillus subtilis* by biochemical identification method. SEM micrographs taken confirmed the formation of biofilm. It was 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 result indicated the presence of a macromolecular complex constituting of carbohydrate, protein, lipids and nucleic acids.

1. INTRODUCTION

A biofilm is a complex aggregation of micro−organisms growing on a solid substrate. Biofilms occur in a range of everyday situations, from pipe and ship fouling to dental caries. Biofilms grow in a three stage process. The initial stage includes the attachment of bacteria to the substratum. Bacterial growth and division then leads to the colonization of the surrounding area and the formation of the biofilm. Bacteria do not act individually to form biofilms, but congregate into long chains to help initiate the early stages of biofilm formation. Mature biofilms are a complex heterogenous structure of dormant and actively growing bacteria colonies along with further enzymes, excretory products and small channels forming part of the overall structure. The major features that distinguish biofilm forming bacteria from their planktonic counterparts are their surface attachment ability, high population density, extracellular polymeric substances (EPS) slime and a wide range of physical, metabolic and chemical heterogeneities (Beer and Stoodley, 2006). It is now recognized that biofilm formation is an important aspect of many diseases, including native valve endocarditis, osteomyelitis, dental caries, middle ear infections, medical device-related infections, ocular implant infections, and chronic lung infections in cystic fibrosis patients (Donlan and Costerton, 2002). Biofilms can tolerate antimicrobial agents at concentrations of 10–1000 times than that needed to kill genetically equivalent planktonic bacteria, and are also extraordinarily resistant to phagocytosis, making biofilms extremely difficult to eradicate from living hosts (Lewis, 2001). The formation of a biofilm in turn also alters the microenvironment of its own inhabitants which then leads to additional alterations in gene expression and further maturation of the biofilm, and so on. Four potential incentives behind the formation of biofilms by bacteria during infection are proposed (Jefferson, 2004): (i) protection from harmful conditions in the host (defense), (ii) sequestration to a nutrient-rich area (colonization), (iii) utilization of cooperative benefits (community), (4) biofilms normally grow as biofilms and planktonic cultures are an in vitro artifact (biofilms as the default mode of growth).

Oral microbial-plaque communities are biofilms composed of numerous genetically distinct types of bacteria that live in close juxtaposition on host surfaces. These bacteria communicate through physical interactions called coaggregation and coadhesion, as well as other physiological and metabolic interactions. Streptococci and actinomyces are the major initial colonizers of the tooth surface, and the interactions between them and their substrata help establish the early biofilm community. Formation of dental plaque takes place in a sequential manner leading to a structurally and functionally organized, species-rich microbial community (Marsh, 2004). Distinct stages in plaque formation include: acquired pellicle formation, reversible adhesion involving weak long-range physicochemical interactions between the cell surface and the pellicle followed by stronger adhesin-receptor mediated attachment which ultimately leads to co-adhesion resulting in secondary attachment to already attached cells (Koelenbrander et al., 2000). This complex stage on further multiplication leads to biofilm formation. The composition of oral biofilm to a great extend depends on balance among the component species in spite of regular minor environmental stresses, e.g., from dietary components, oral hygiene, host defenses, diurnal changes in saliva flow, etc. This stability or balance (termed *microbial homeostasis*) does not depend on any biological indifference among the resident organisms, but arises due to a balance imposed by numerous microbial interactions, which include both synergism and antagonism (Marsh et al., 1989).

Dental caries is formed when the balance inside mouth is disturbed. If the proportion of acidogenic and aciduric (acid-tolerating) bacteria increases it leads to demineralization of enamel by rapid metabolism of dietary sugars to acid, resulting into a low pH. Thus, favourable micro environment is created for these organisms enhancing their growth and multiplication whereas most species associated with enamel health are sensitive to acidic environmental conditions (Fig. 1). Thus, oral biofilm can be studied as an ecological plaque signifying that disease can be prevented not only by targeting the pathogenic microbes directly by antimicrobial or anti-adhesive strategies, but also by regulating the selection pressures responsible for their growth and multiplication (Marsh, 2003).

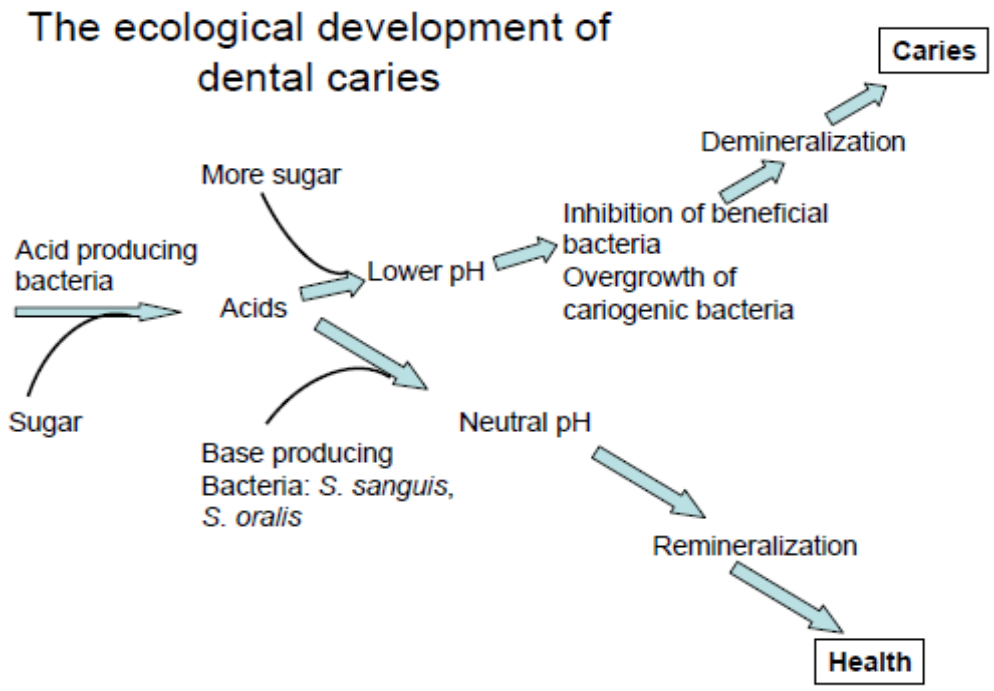


Fig 1. Schematic representation of ecological development of dental caries.

This project focuses on response of oral biofilm forming bacteria to various stress conditions like pH, temperature, sugar, salinity, metal and antibiotics. An attempt has also been made to study the chemotactic movement of isolated species towards various sugars and salts and characterization of biofilm matrix (EPS).

2. REVIEW OF LITERATURE

2.1 Biofilm formation

The survival of bacteria in rapidly changing environment is due to their flexibility in gene expression which is an outcome of mutation, recombination within genes, acquisition of new genetic material and mutation. Genetic adaptation bestows upon them the ability to be creative in their regulatory process and enables them to survive transitioning between life in the environment and in the human host. They can also adapt to sudden changes in nutrient availability and to primary and secondary host immune defenses. One particularly important and relevant example of bacterial adaptation through systematized gene expression is the ability to grow as part of a sessile, exopolymer-enshrouded community referred to as a biofilm (Jefferson, 2004). They are defined as communities of microorganisms adhered to a surface. They are known to undergo remarkable changes during their transition from planktonic stage to cells that are part of a complex, surface-attached community. New phenotypic characteristics are developed by biofilm forming bacteria due to these changes and are known to occur in response to many environmental signals (O'Toole et al., 2000).

Biofilm formation act as a defensive method during various stress conditions Biofilms are resistant to physical forces such as the shear forces produced by blood flow and the washing action of saliva. Organisms within biofilms can withstand nutrient deprivation, pH changes, oxygen radicals, disinfectants, and antibiotics better than planktonic organisms. Biofilms are also resistant to phagocytosis, and the phagocytes that attempt an assault on the biofilm may actually do more harm to surrounding tissues than to the biofilm itself, making biofilms extremely difficult to eradicate from living hosts (Lewis, 2001). The chronic nature of certain infections is inarguably due to the development of a resilient biofilm. The invulnerability of biofilms is not completely understood but is likely dependent upon a number of biofilm-specific characteristics including slow growth and physiologic heterogeneity of the inhabitants. Another important trait that fortifies biofilm resistance is the sticky matrix which may contain

DNA and other polymers but in general, is predominantly composed of exopolysaccharides (Jefferson, 2004).

Bacteria forms biofilms largely to remain in a favourable niche (Jefferson, 1994). Humans and other animals have developed intricate immune systems for one critical reason: microorganisms are continually trying to inhabit their bodies. The body, or at least parts of it, is nutrient rich and relatively stable with respect to water content, oxygen availability, and temperature. Consequently, there is a never-ending race between the development of the host immune system and the progression of bacterial strategies to evade it. In some cases, a compromise has been made, and as such, the body is inhabited by a large number of commensals, many of which exist as biofilms. As the body is obviously an appealing place for bacteria to live, it may be that the primary motivation for switching to the biofilm mode of growth is to remain fixed. Bacteria have a number of strategies to ensure that they remain fixed in the human body. Bacterial surface proteins that bind to host extracellular matrix proteins and often play a key role in initial adherence of bacteria to solid surfaces within the host (Patti et al., 1994).

2.2 Oral Biofilm

Oral bacterial species mostly lack an environmental niche and are found almost exclusively within the mouth (Rachid et al., 2000). For these bacteria, planktonic growth would cause them to be quickly washed away by saliva, swallowed and destroyed within the acidic juices of the stomach. These bacteria likely spend the majority of their natural existence growing as a biofilm.

Dental plaque development is a natural phenomenon and is known to contribute to the host defence by preventing colonization by exogenous species. The bacteria constituting the oral biofilm varies at distinct sites as a result of the inherent biological and physical properties at these sites. However, the balance of the bacterial population shows a shift in disease condition like dental caries (Marsh, 2003).

The pattern of plaque biofilm development can be divided into three phases:

1. Attachment of bacteria to a solid surface;
2. Formation of microcolonies on the surface; and
3. Formation of the mature, subgingival plaque biofilms

The initial attachment of bacteria begins with pellicle formation. The pellicle is a thin coating of salivary proteins that attach to the tooth surface within minutes after a professional cleaning. The pellicle acts like double-sided adhesive tape, adhering to the tooth surface on one side and on the other side, providing a sticky surface that facilitates bacterial attachment to the tooth surface. Following pellicle formation, bacteria begin to attach to the outer surface of the pellicle. Bacteria connect to the pellicle and each other with hundreds of hairlike structures called fimbriae. Once they stick, the bacteria begin producing substances that stimulate other free floating bacteria to join the community. Within the first 2 days in which no further cleaning is undertaken, the tooth's surface is colonized predominantly by gram positive facultative cocci, which are primarily streptococci species. It appears that the act of attaching to a solid surface stimulates the bacteria to excrete an extracellular slime layer that helps to anchor them to the surface and provides protection for the attached bacteria. Microcolony formation begins once the surface of the tooth has been covered with attached bacteria. The biofilm grows primarily through cell division of the adherent bacteria, rather than through the attachment of new bacteria. Next, the proliferating bacteria begin to grow away from the tooth. Plaque doubling times are rapid in early development and slower in more mature biofilms. Bacterial blooms are periods when specific species or groups of species grow at rapidly accelerated rates (Gehrig, 2003).

A second wave of bacterial colonizers adheres to bacteria that are already attached to the pellicle. Coaggregation is the ability of new bacterial colonizers to adhere to the previously attached cells. The bacteria cluster together to form sessile, mushroom-shaped micro colonies that are attached to the tooth surface at a narrow base. The result of coaggregation is the formation of a complex array of different bacteria linked to one another. Following a few days of undisturbed plaque formation, the gingival margin becomes inflamed and swollen. These inflammatory changes result in the creation of a deepened gingival sulcus. The biofilm extends into this subgingival region and flourishes in this protected environment, resulting in the formation of a mature subgingival plaque biofilm (Fig. 2). Gingival inflammation does not appear until the biofilm changes from one composed largely of gram-positive bacteria to one containing gram-negative anaerobes. A subgingival bacterial microcolony, predominantly composed of gram-

negative anaerobic bacteria, becomes established in the gingival sulcus between 3 and 12 weeks after the beginning of supragingival plaque formation. Most bacterial species currently suspected of being periodontal pathogens are anaerobic, gram-negative bacteria (Kroes et al., 1999).

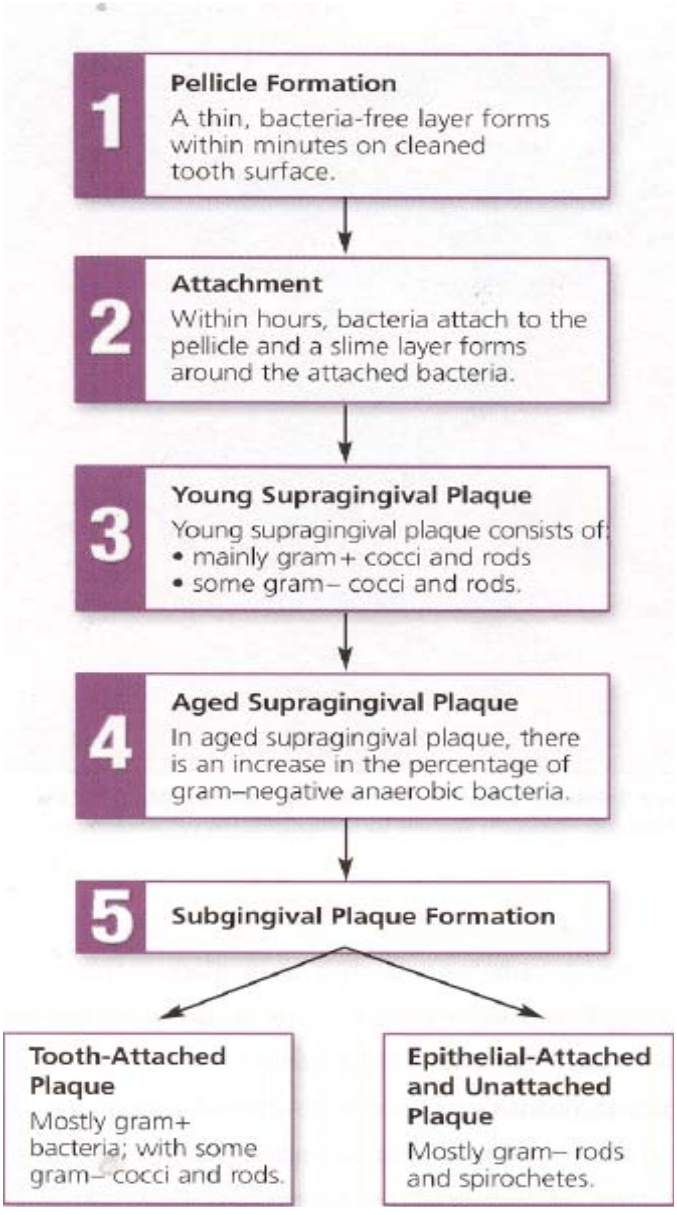


Fig 2. Figure showing phases of plaque formation (Gehrig, 2003).

It has been reported that in dental caries, there is a shift favoring dominance by acidogenic and acid-tolerating species such as streptococci and lactobacilli. The inhibition of biofilm development including prevention of attachment of cariogenic bacteria, manipulation of cell signaling mechanisms, delivery of effective antimicrobials or enhancement of the host defenses could be the strategies to prevent dental caries. The study of the factors that enable the cariogenic bacteria to escape from the normal homeostatic mechanisms that restrict their growth in plaque and out compete the organisms associated with health could be helpful in inhibiting the formation of caries. The consistent low pH in dental caries favours the selection of acid producing bacteria Therefore, non fermentable artificial sugars, metabolic inhibitors or stimulation of saliva flow can bring about the suppression of sugar catabolism and acid and restore the balance favouring normal microflora (Marsh, 2006).

Bacteria involved in dental caries may be found naturally in dental plaque but being present in a very small proportion they are only weakly competitive at neutral pH. Thus, with a conventional diet the process of mineralization and demineralization is in equilibrium. However, if the intake of fermentable carbohydrate is more there is more acid production which in turn favours acid tolerating species leading to demineralization of enamel. It has two effects on overall microbial ecology. Firstly, low pH favours the survival of acid producing bacteria and secondly it shifts the balance towards demineralization (Sansone et al., 1993). These bacteria could be responsible for some of the initial stages of demineralization. Thus, oral biofilm can be studied as an ecological plaque signifying that disease can be prevented not only by targeting the pathogenic microbes directly by antimicrobial or anti-adhesive strategies, but also by regulating the selection pressures responsible for their growth and multiplication (Marsh, 2003).

Dental caries is formed when the balance inside mouth is disturbed. If the proportion of acidogenic and aciduric (acid-tolerating) bacteria increases it leads to demineralization of enamel by rapid metabolism of dietary sugars to acid, resulting into a low pH. Thus, favourable micro environment is created for these organisms enhancing their growth and multiplication whereas most species associated with enamel health are sensitive to acidic environmental conditions (Loesche, 1986 and Becker, 2002). The one

which are capable of fermenting acids are capable of demineralizing enamel. The most common bacteria associated with caries is mutans streptococci but the association is not unique as caries can occur in the apparent absence of these species and also these bacteria can be present without evidence of detectable demineralization (Marsh, 1989), i.e., some acidogenic, non-mutans streptococci can also be associated with disease (Bowden, 1990 and Marsh, 1999).

The parameters which are of significant importance in regulating homeostasis in the mouth include mainly the integrity of the host defenses (including saliva flow) and the composition of the diet. Consumption of food with high fermentable sugar content have greater proportions of mutans streptococci and lactobacilli in plaque. Antimicrobial peptides are though recognized as important components in controlling microbial populations in the mouth, much is not known about their role in regulating the resident microflora at sites in the body, but risk of caries is known to increase with reduction in their activities. It has been reported that their role is complex because they are multi-functional and have more than a mere antimicrobial action, e.g., by linking the innate and adaptive arms of the immune response (Devine, 2003).

3. OBJECTIVES

Oral bacteria in plaque do not behave as individuals rather function as a co-ordinated, metabolically integrated and well organized microbial community which shows concerted behaviour. A better understanding of the existence of oral bacteria as a biofilm rather than individuals can lead to effective control strategies. Keeping this in mind the present study aimed to fulfil the following objectives:

1. Isolation of oral bacteria.
2. *In vitro* screening for biofilm formation.
3. Biochemical identification of bacterial strains.
4. Planktonic growth kinetics and kinetics of biofilm formation.
5. Study of chemotactic response to different sugars and metals.
6. Study of biofilm formation under stress conditions.
7. Characterization of biofilm matrix (EPS).

4. MATERIALS AND METHODS

4.1 Isolation of oral bacteria

4.1.1 Sample Collection

Sample was collected from mouth by swabbing across the gingival and subgingival region as well as from the roof and floor of the bucal cavity. The samples were collected from three persons and were inoculated in Nutrient broth (HiMedia, India) and viable cells were enumerated.

4.1.2 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 spread on to Nutrient agar (HiMedia) plates and incubated overnight at 37ºC. After 12 -18 hours incubation the number of viable colonies were counted using total viable plate count method (Prescott and Harley, 2002).

C.F.U/mL original sample = Number of colonies /plate x (1/mL aliquot plated) x dilution factor

4.2 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 was checked in Lauria Bretani (LB) (HiMedia) 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 microtitre plate biofilm assay (Mack et al., 1994 and O'Toole et al., 1999).

4.2.1 The Tube assay

Qualitative assessment of biofilm formation was determined by the tube staining assay following Christensen et al., LB broth (1mL) was inoculated with 100µl of overnight culture broth and incubated for 24, 48 and 72 hours at 37°C. The tubes were decanted and washed with Phosphate Buffer Saline (PBS) (pH 7.3) and dried. Staining of dried tubes was done with 0.1% crystal violet. Excess stain was removed by washing the tubes with deionized water. Biofilm formation in tubes was then observed.

Formation of biofilm was confirmed with the presence of attachment (visible film) on the wall and bottom of the tube. However, the liquid interface did not indicate biofilm formation (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.

4.2.2 The Microtitre plate assay

The isolates were screened for their ability to form biofilm by microtitre plate method. The cultures were screened in three different media for biofilm formation, *viz,* i.e, Nutrient broth, LB broth and Basal media (HiMedia, India) supplemented with glucose. Isolates from fresh agar plates were inoculated in respective media and incubated for 18 hour at 37^{o}C in static condition. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates (Tarson, Kolkata, India) wells were filled with 200µl aliquots of the diluted over night cultures (100 times diluted) and broth without culture was used as control.

The plates were incubated for 24 hours, 48 hours and 72 hours at 37°C to observe biofilm formation. After respective incubation period content of each well was gently removed by slightly tapping the plates. The wells were then washed with phosphate buffer saline (PBS pH 7.3) to remove free-floating 'planktonic' bacteria. The plates were then stained with 0.1% (w/v) crystal violet solution. Excess stain was washed off thoroughly with 95% ethanol and plates were kept for drying. Optical density (OD) of of the wells was determined with a micro ELISA auto reader (Perkin Elmer) at wavelength of 570 nm. These OD values were considered as an index of attachment to surface and

forming biofilms. The experiment was performed in triplicate and repeated three times and the mean OD value was considered.

4.3 Biochemical identification of bacterial strains

Biochemical identification of the selected strains was performed by physical and biochemical characterization

4.3.1 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 immersion to determine the morphological characteristics 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.

4.3.2 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.

4.4 Growth kinetics

Growth kinetics of the isolated strains was studied in planktonic and biofilm formation

4.4.1 Planktonic Growth Kinetics

5 ml of LB broth was inoculated with 50µl of overnight broth culture of the test organism and initial OD was taken at 630 nm. The culture was incubated at 37ºC at 180rpm orbital shaking incubator and the OD was measured at an interval of every 30mins. A graph of Time Vs Absorbance was plotted to determine the planktonic growth kinetics of the isolated strains.

4.4.2 Kinetics of biofilm formation

Overnight broth culture of the test organisms were inoculated into 5 sets of 96 well microtitre plates taking 100µl each of 1:100 dilution and the absorbance was read out using ELISA auto reader at 570 nm at an interval of every 2 days following similar staining procedures which was used for screening. The OD values were plotted against time interval to determine biofilm formation kinetics of the isolated strains.

4.5 Chemotaxis

Chemotactic response of the isolated bacterial strains to different sugars (Fructose, Sucrose and Arabinose) and metals (Ferrous sulphate, Calcium chloride and

Lead chloride) was studied 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 5 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.

4.6 Stress Response Study

The isolated strains were studied for biofilm formation under various stress conditions like sugar, salinity, temperature, pH, metal and antibiotics.

4.6.1 Sugar stress

Stock solution of five sugars, i.e.; glucose, sucrose, arabinose, fructose and xylose having concentration of 10x 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 taking sugars individually as the only carbon source in basal media. 900 µl of media (containing respective sugar in 1% conc.) was inoculated with 100µl of overnight broth culture of the bacterial strains. 100µl of the same concentration of inoculum was inoculated in 96 well microtitre plates and was incubated at 37ºC for 72 hours. After incubation, the contents 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 and allowed to dry. The OD of the adherent biofilm was taken using a micro ELISA auto reader at 570nm.The tubes were stained in similar manner and results obtained with different sugars were compared.

4.6.2 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. 1 ml of media with repective salt concentration was inoculated with overnight culture in 1:10 dilution. 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.

4.6.3 Temperature stress

1 ml of media was inoculated with overnight culture in 1:10 dilution. 200µl of same inoculum concentration was inoculated into 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.

4.6.4 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.

4.6.5 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.

4.7 Characterization of biofilm matrix (EPS)

4.7.1 EPS extraction

Biofilm samples were placed on ice for thawing and centrifuged at 15000× g for 20 minutes. The biofilm pellets were resuspended in about 30 ml of cold sulfuric acid, i.e., 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 4ºC for 3 hours with

occasional stirring before centrifugation at 15000 × g for 20 minutes. The resulting supernatant containing total EPS was used for further analysis (Jiao et al., 2010).

4.7.2 Fourier Transform Infrared (FTIR) analysis

Fourier transform infrared (FTIR) 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).

4.7.3 Chemical analysis

The total protein concentration was measured by adding 12% Trichloroacetic acid (TCA) to EPS solution and the mixture was incubated on ice for 30 minutes and then centrifuged at 15,000×g for 20 minutes. The TCA precipitates were then 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 method with BSA as the calibration standard.

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 and then DNA was recovered by centrifuging at 17,500×g for 20 minutes 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 spectrophotometer (Elico® Double Beam).

3.7.4 Characterization of biofilm by Scanning Electron Microscopy (SEM)

The bacterial strains were inoculated in tubes with glass slides of dimension 1x1cm inside the tubes. They were inoculated in static condition at 37°C for 72hrs. The slides were then air dried and observed under SEM at various resolutions.

5. RESULTS AND DISCUSSION

5.1.1 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 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}

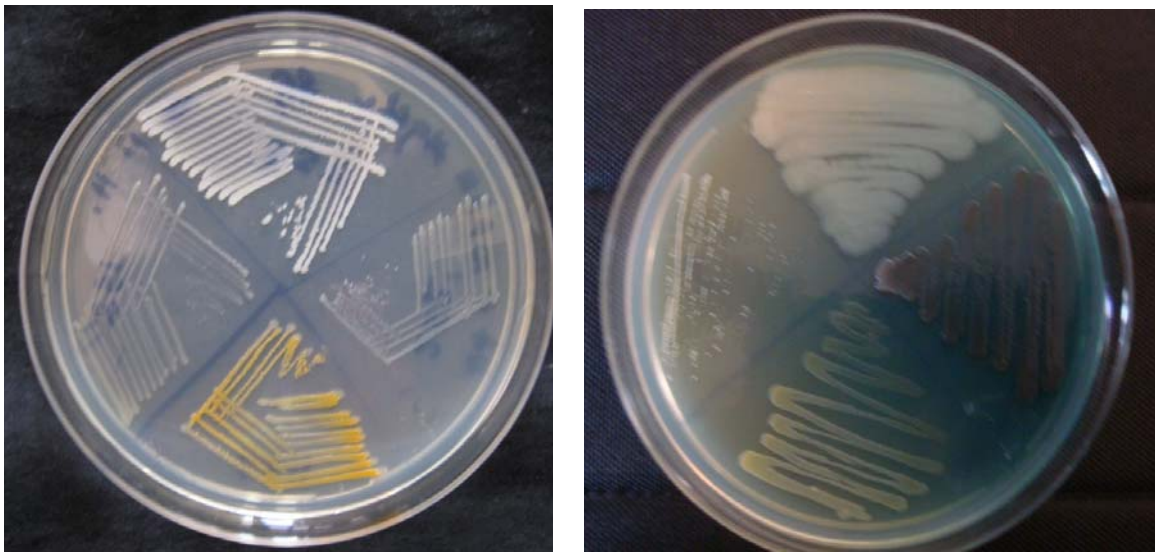
10 bacterial strains with observable difference in colony morphology were randomly selected from initial spread plate and restreaked (Fig. 3).

(a) (b) (c)

Fig. 3 (a)-(c) Spread plate culture of dental plaque bacteria.

5.1.2 Isolation of strains:

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



(a) (b)

Fig. 4 (a),(b) Pure cultures of the isolated strains.

5.2 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.

5.2.1Tube assay:

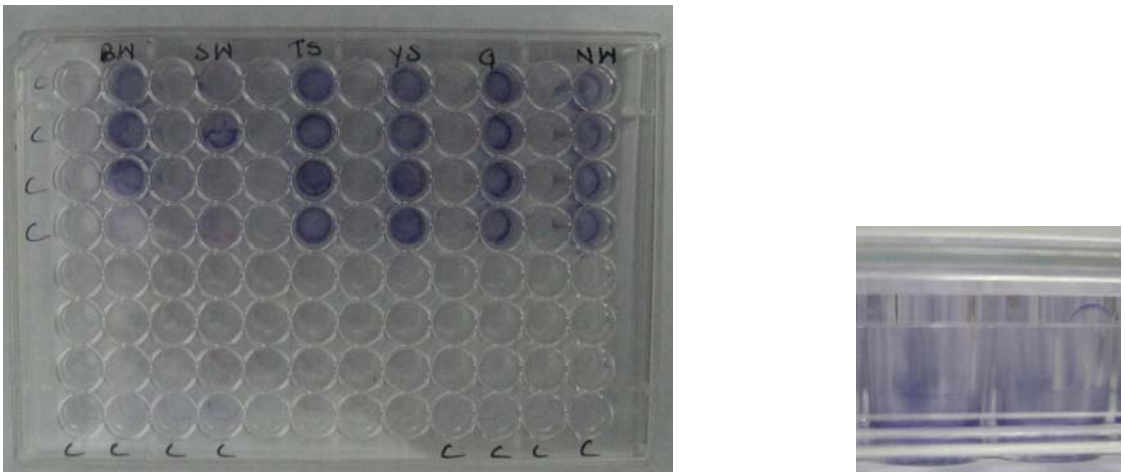
The tubes when stained with crystal violet showed biofilm attachment. (Fig. 5)

(a) **(b)** **(c)**

Fig. 5 (a) –(c) Stained tubes showing biofilm attachment.

5.2.2 Microtitre plate assay: (Primary screening)

The mictrotiter plate assay showed attachment at the bottom of the wells containing biofilm sample. The samples were added to alternative wells (2,4,6,8,10 and 12) and control was kept in well 1 (Fig. 6). In result table the yellow and grey highlighted readings indicate absorbance reading for control and samples respectively.



(a) **(b)**

Fig. 6 Three day biofilm screening of the isolated strains.

Absorbance reading of 3 day and 5 day biofilm screening are shown in Table. 2-3.

Table 2. 3 day biofilm screening.

	1	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

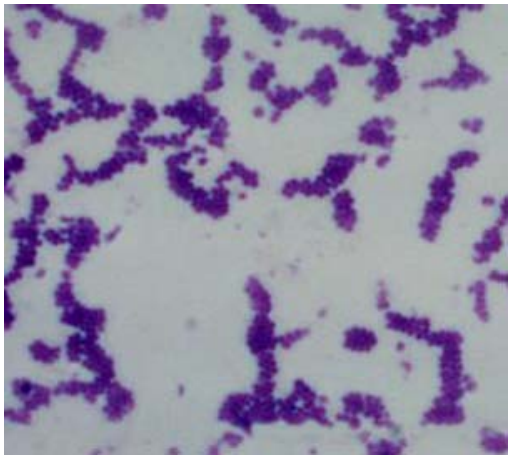
Table 3. 5 day Biofilm screening.

	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

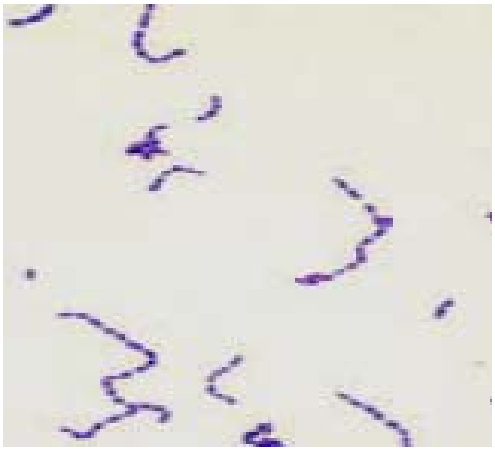
5.3 Characteization of Bacterial Strains

5.3.1 Physical characterization:

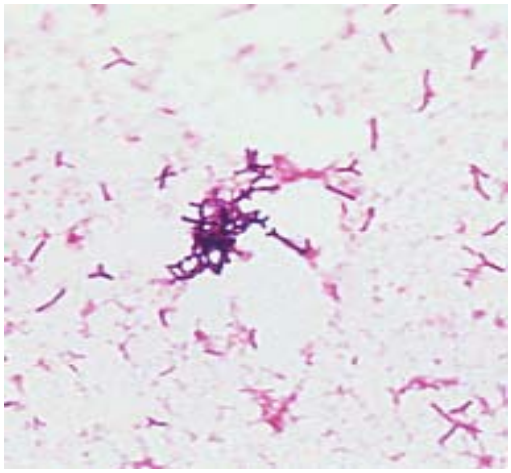
The isolated bacterial species BW, SW, TS, YS, G and NW were morphologically characterized by gram staining and were found be Gram +ve staphylococcus, Gram +ve staphylococcus, Gram +ve streptococci, Gram +ve streptococci, Gram –ve bacilli and Gram +ve bacilli respectively (Fig. 7 a-e). Motility test for all bacterial strains were done on SIM media and all were found to be motile (Fig. 8-10).



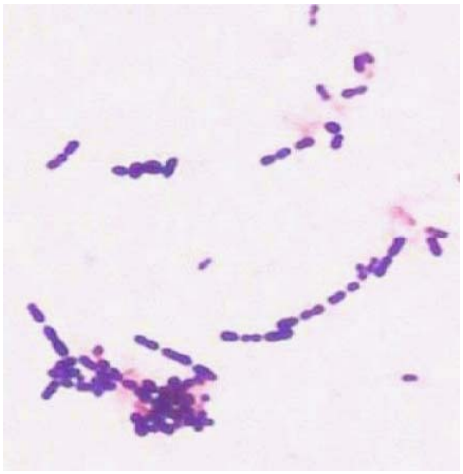
a) Staphylococcus aureus



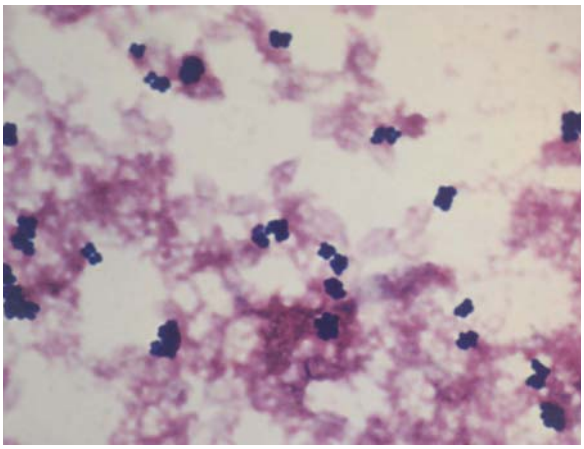
b) Streptococcus mitis



c) Bacillus subtilis



d) Streptococcus oralis



e) Staphylococcus salivarius

Fig. 7 Gram staining pictures of the isolated bacterial strains.

5.3.2 Biochemical identification:

The six bacterial species were biochemically using HiMedia rapid biochemical identification kit (Fig. 8) 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. However, G strain could not be identified.

Fig. 8 Biochemical identification of isolated bacterial strains.

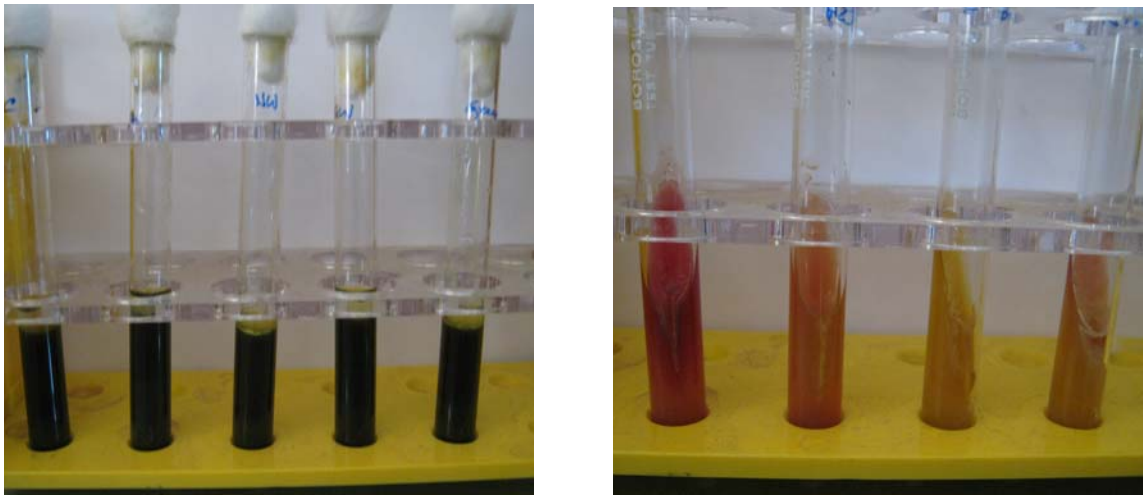


Fig. 9(a) Bacterial strains grown in Basal media (b) Triple sugar utilization test of strains isolated.

Fig. 10 Motility test of isolated bacterial strains in SIM media.

5.4 Growth kinetics

5.4.1 Planktonic growth kinetics

The isolated strains in planktonic form showed the following pattern of growth (Fig. 11-16).

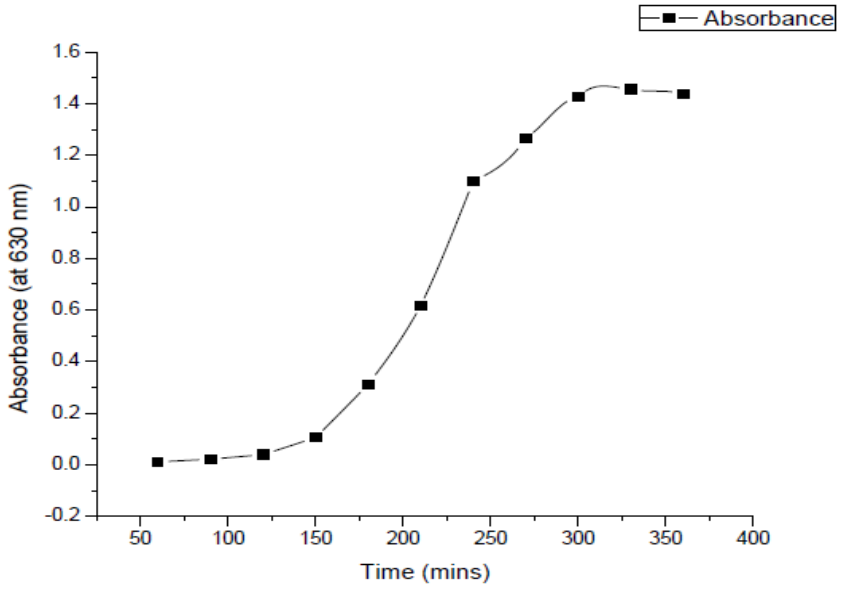


Fig. 11 Planktonic growth curve of YS.

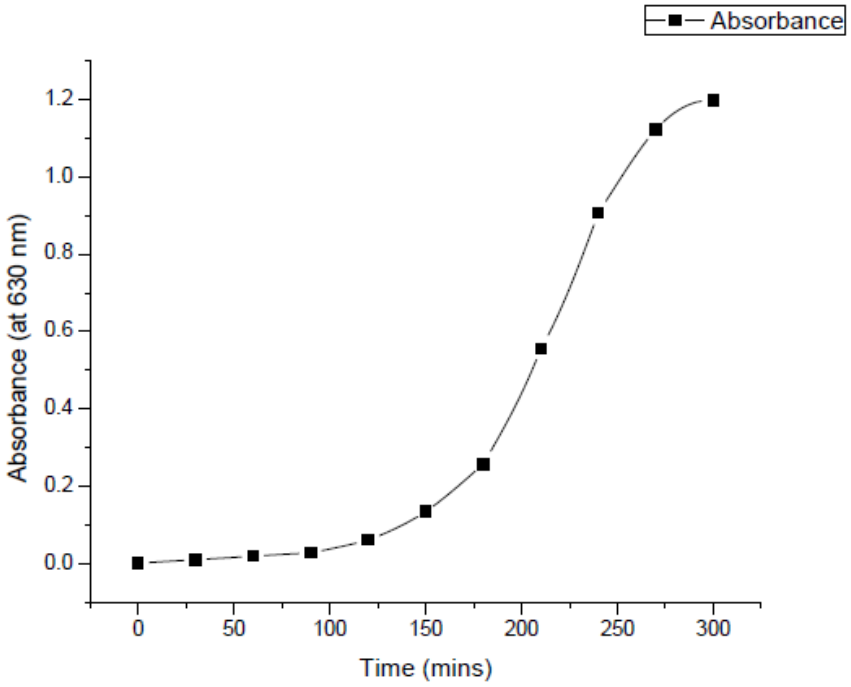


Fig. 12 Planktonic growth curve of TS.

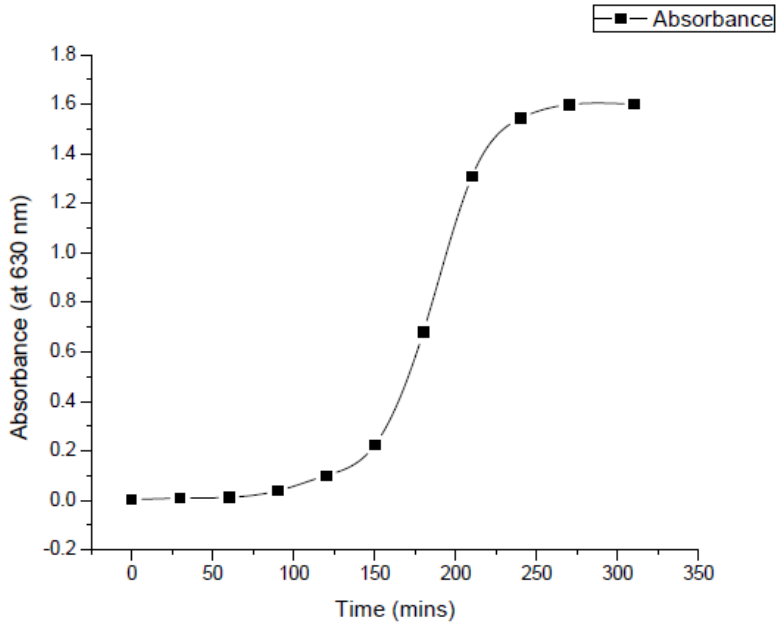


Fig. 13 Planktonic growth curve of SW.

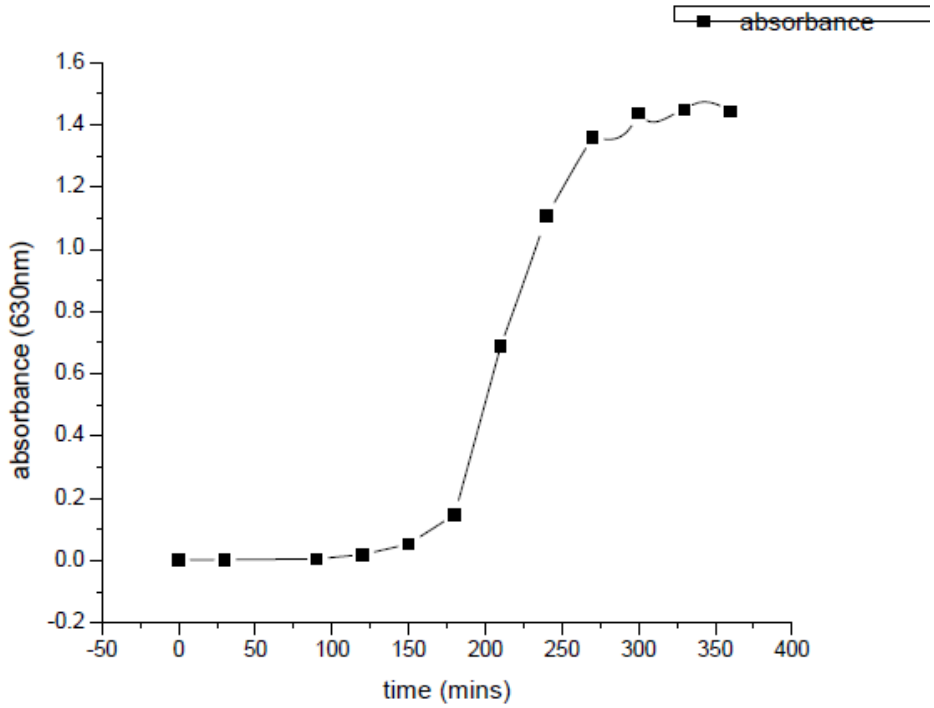


Fig. 14 Planktonic growth curve of NW.

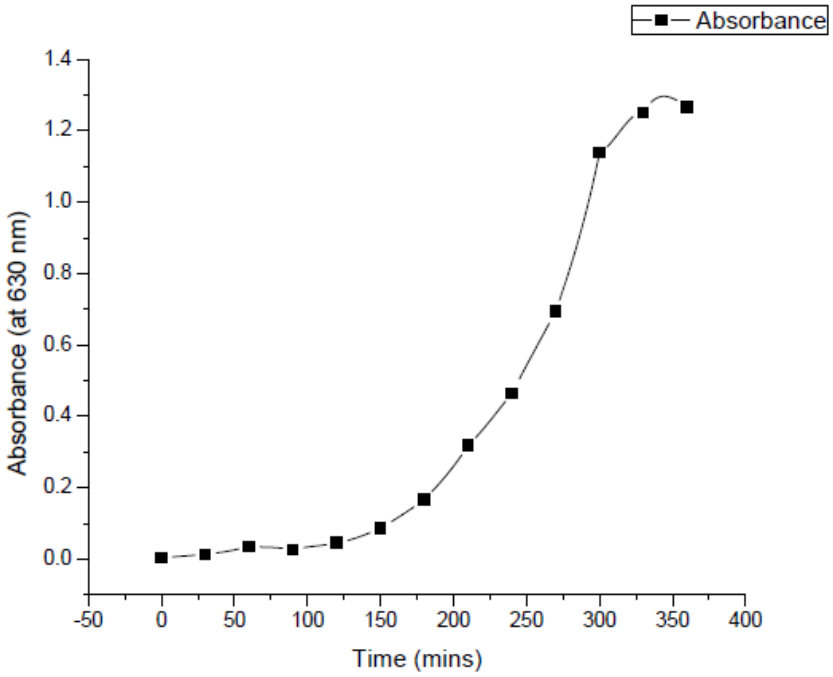


Fig. 15 Planktonic growth curve of G.

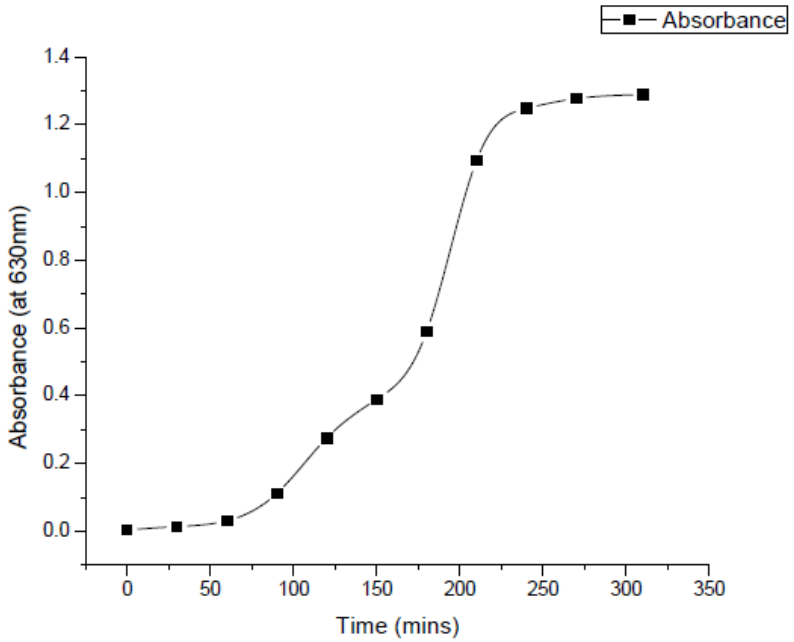


Fig. 16 Planktonic growth curve of BW.

5.4.2 Kinetics of biofilm formation

The biofilm kinetics of strains was also observed. It showed a sharp decline after 7 days due to disintegration. NW, however, showed disintegration after 10 days and BW showed after 5 days (Fig. 17-22).

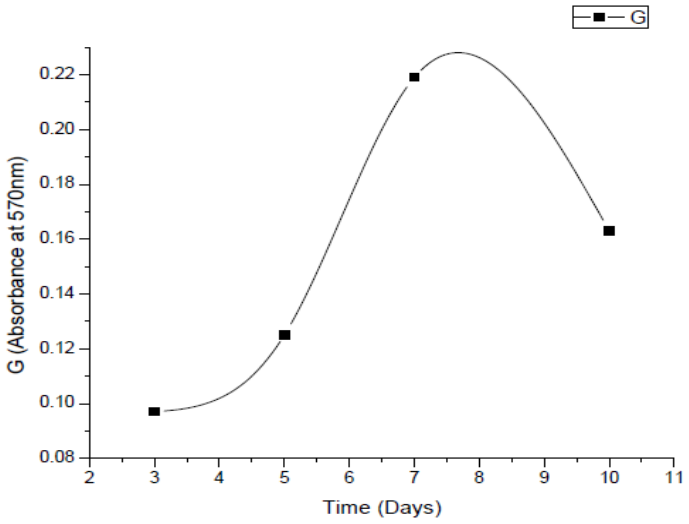


Fig. 17 Biofilm formation kinetics of G.

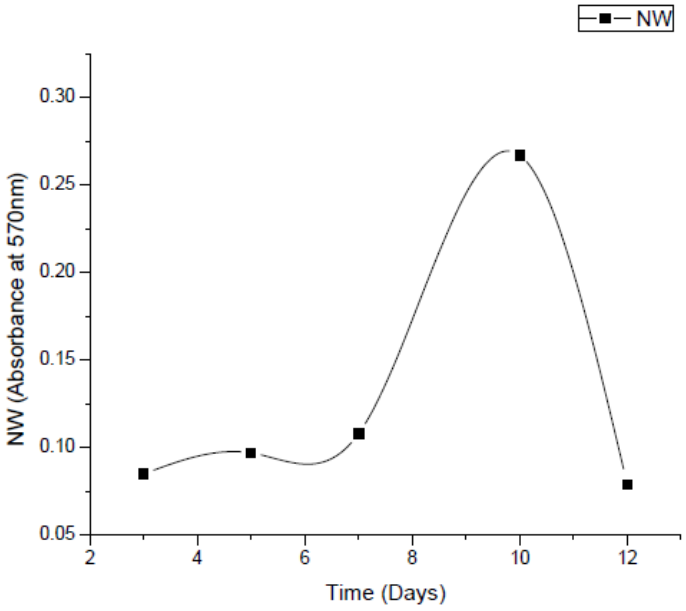


Fig.18 Biofilm formation kinetics of NW.

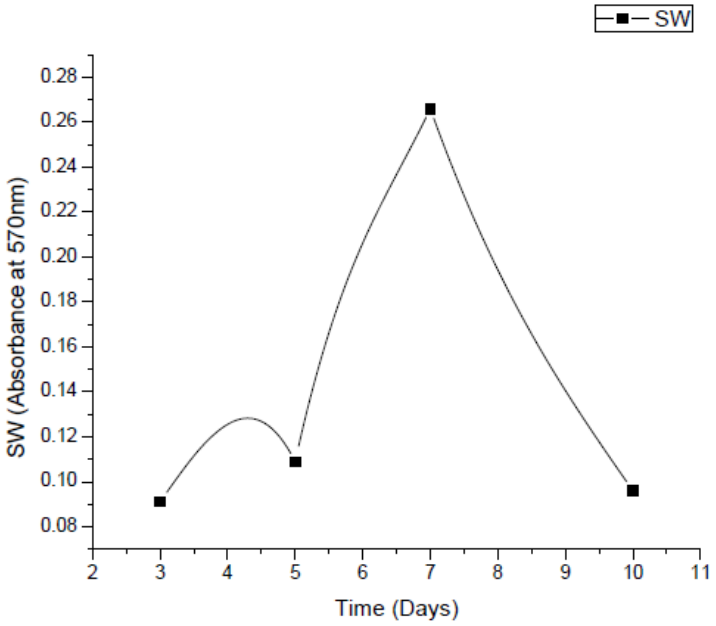


Fig.19 Biofilm formation kinetics of SW.

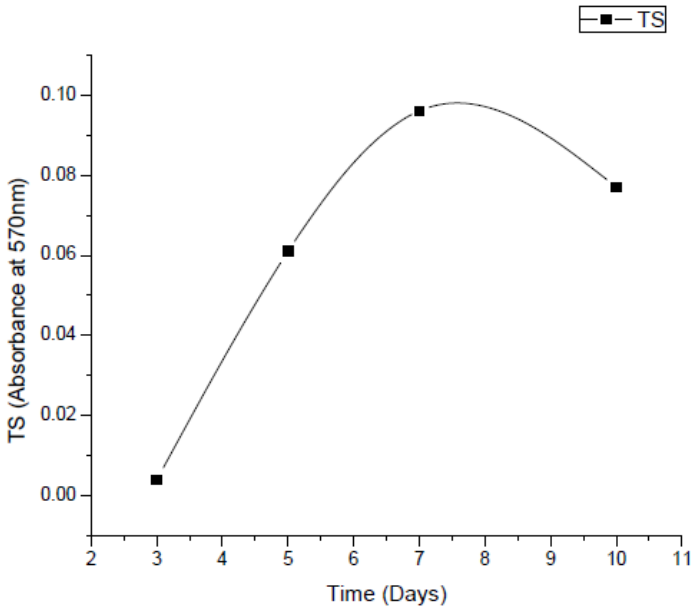


Fig 20. Biofilm formation kinetics of TS.

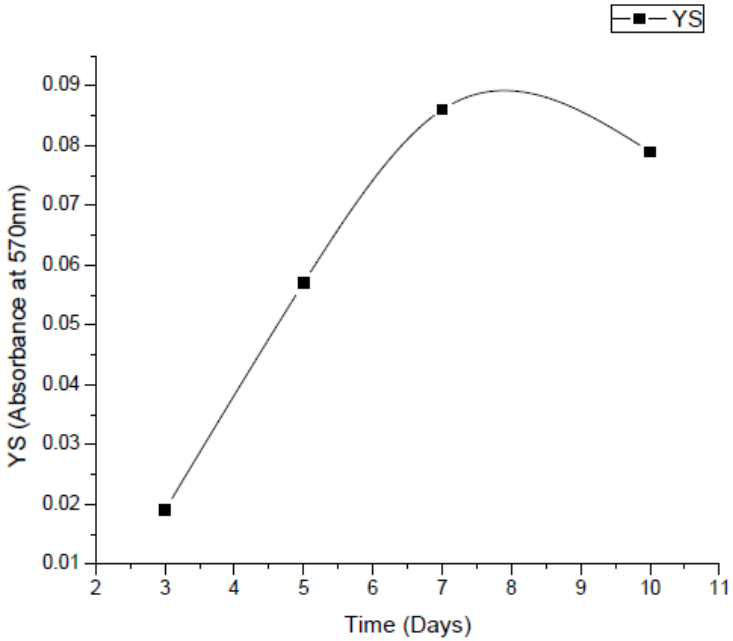


Fig. 21 Biofilm formation in YS.

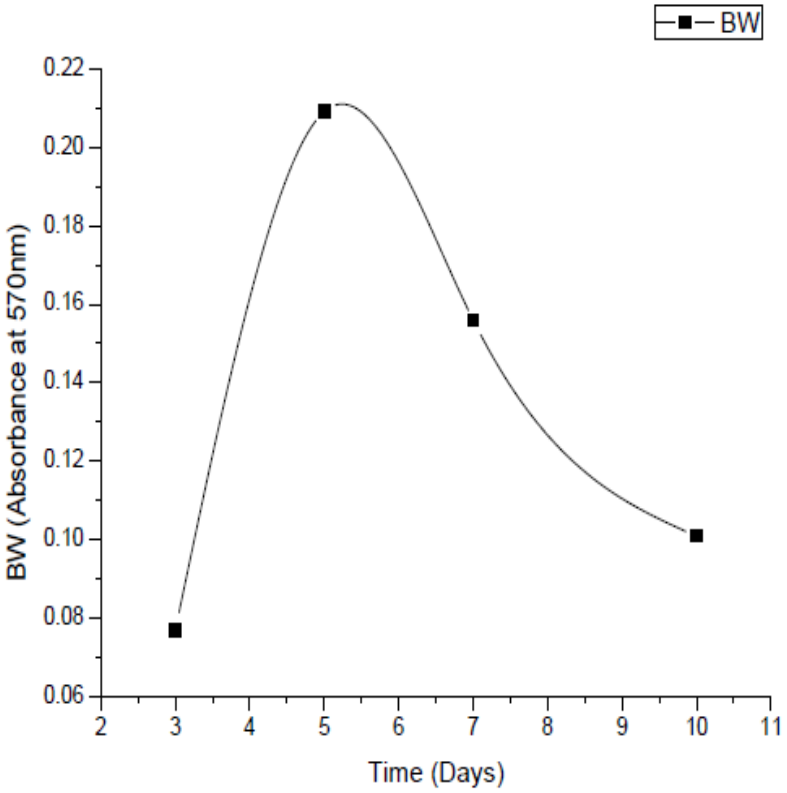


Fig.22 Biofilm formation in BW.

Comparative analysis of planktonic growth kinetics showed that G is comparatively slow growing whereas all other strains showed the normal trend (Fig. 23).

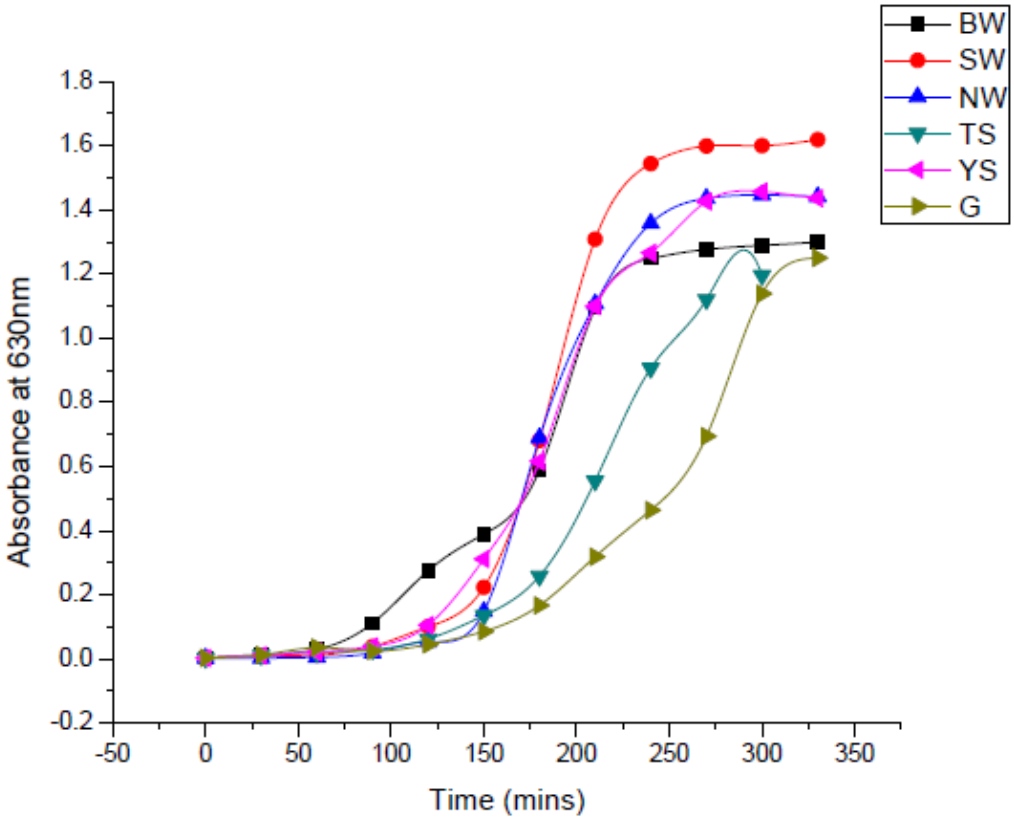


Fig. 23 Comparative analysis of planktonic growth curve of BW, SW, NW, TS, YS, G.

Comparatively analysis of biofilm growth kinetics showed that NW strain secreted EPS for maximum days (10) whereas all other got disintegrated after 7 days except BW which started disintegrating after 5 days only (Fig. 24).

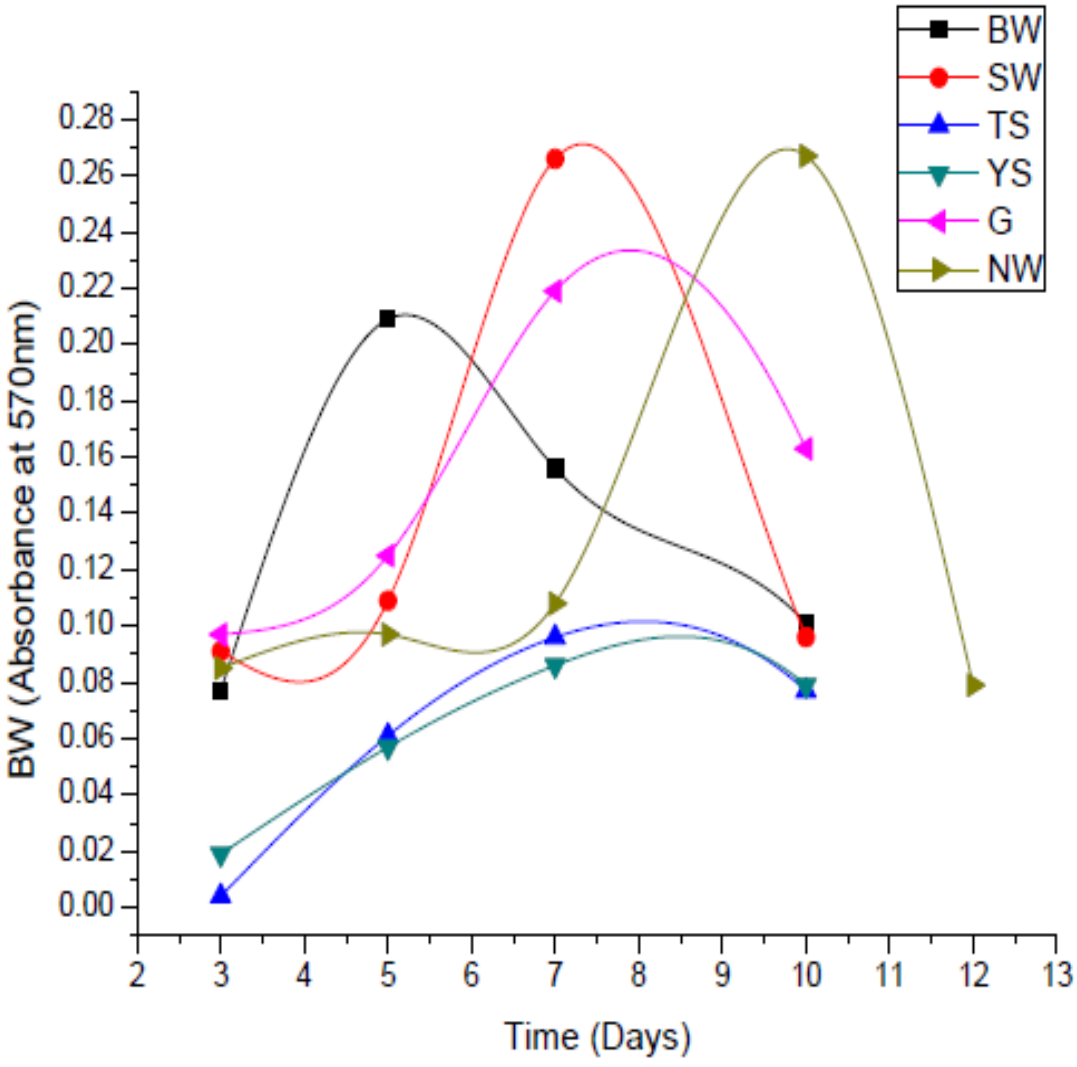


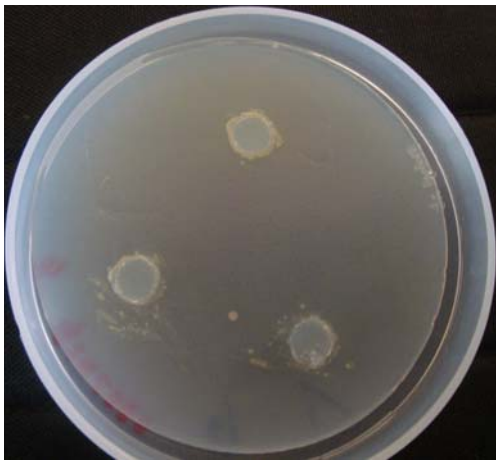
Fig. 24 Comparative analysis of biofilm formation kinetics of BW, SW, TS, YS, G and NW.

5.5 Chemotaxis

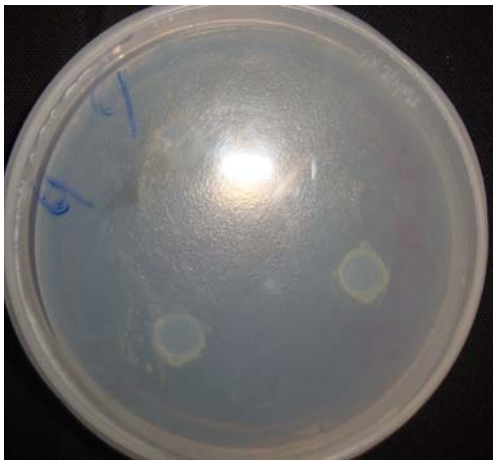
Chemotaxis response of four strains (BW, SW, G and NW) towards sugars (fructose, sucrose and arabinose) and metals ($FeSO_4$, $PbCl_2$ and $CaCl_2$) was studied (Table. 4). It was observed that G and NW did not show chemotactic response towards fructose and sucrose whereas BW and SW showed negative results for arabinose. In case of salts, all the strains showed chemotactic movement towards $CaCl_2$ and none of the strains showed positive result towards $PbCl_2$ and also all but G strain showed chemotaxis towards $FeSO_4$.

Table 4. Chemotactic response.

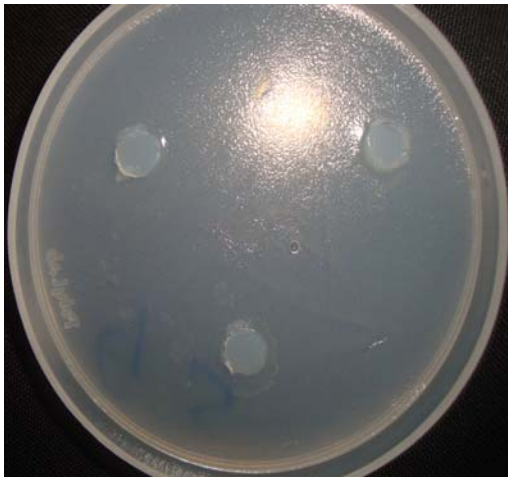
Bacterial strains	Fructose	Sucrose	Arabinose	$FeSO_4$	$PbCl_2$	$CaCl_2$
BW	+	+	-	+	-	+
SW	+	+	-	+	-	+
G	-	-	+	-	-	+
NW	-	-	+	+	-	+



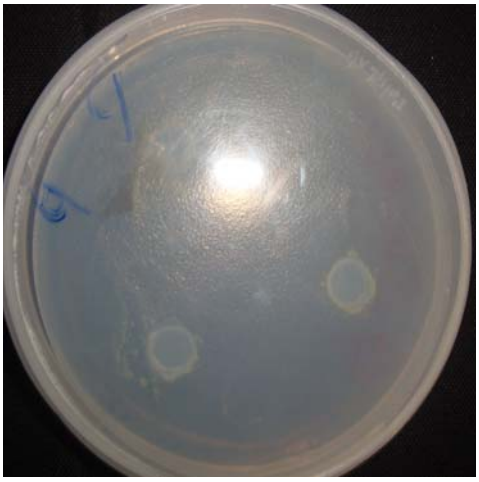
(a)



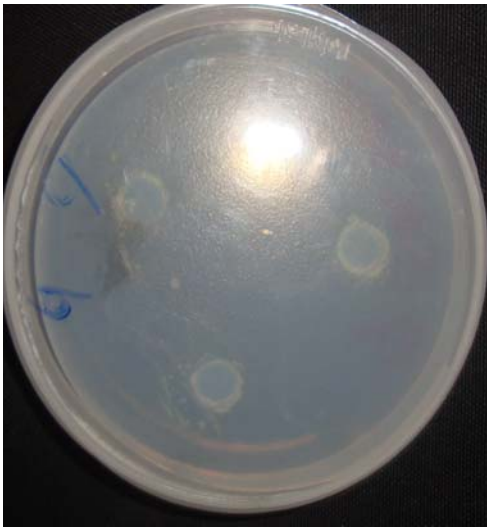
(b)



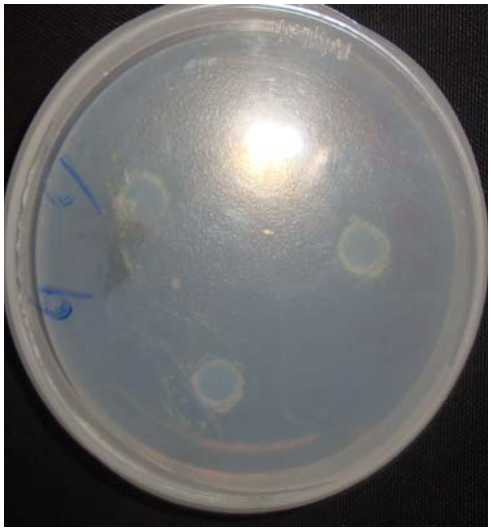
(c)



(d)



(e)



(f)

(g)

Fig. 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).

5.6 Stress response

5.6.1 Sugar stress

Biofilm formation was screened using six different sugars (glucose, sucrose, fructose, arabinose and xylose as the sole carbon source in basal media and the following results were obtained (Table. 5-10) (Fig. 26-29). Fructose was found to be a poor source of carbon as most of the strains showed very less attachment. Glucose and sucrose showed best attachment whereas it was moderate in case of arabinose and xylose. It was also interesting to observe that none of the strains utilized raffinose when it was present as the sole carbon source.

Table 5. Absorbance reading in presence of glucose as the sole carbon source.

	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

Table 6. Absorbance reading in presence of fructose as the sole carbon source.

	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	0.059	0.04	0.041	0.04	0.04
B	0.039	0.050	0.04	0.048	0.039	0.046	0.041	0.073	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

Fig. 26 Tube assay for biofilm screening in presence of fructose.

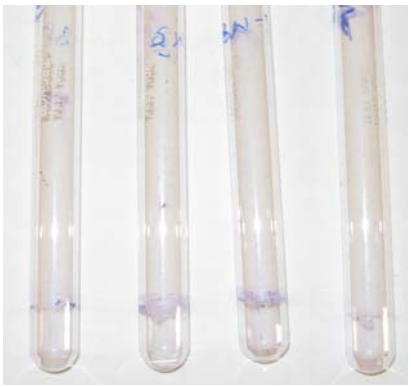


Fig. 27 Tube assay for biofilm screening in presence of glucose.

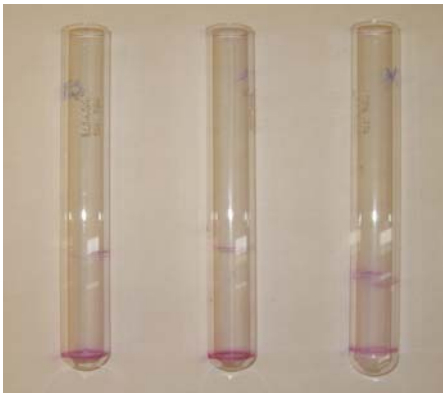


Fig. 28 Biofilm screening in presence of xylose.

Table. 7 Absorbance reading in presence of xylose as the sole carbon source.

	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

Table 8. Absorbance reading in presence of sucrose as the only source of carbon.

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.052	0.04	0.059	0.04	0.048	0.04	0.046	0.04	0.040	0.04	0.041
B	0.041	0.059	0.041	0.057	0.041	0.048	0.039	0.046	0.041	0.041	0.039	0.040
C	0.041	0.056	0.041	0.059	0.04	0.049	0.039	0.044	0.04	0.041	0.039	0.041
D	0.04	0.059	0.04	0.062	0.041	0.049	0.041	0.046	0.039	0.040	0.04	0.040
E	0.041	0.04	0.041	0.04	0.04	0.04	0.04	0.04	0.039	0.04	0.041	0.040
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

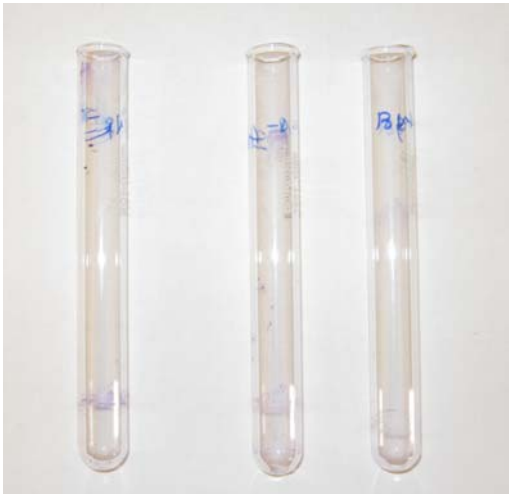


Fig. 29 Biofilm screening in presence of xylose.

Table 9. Absorbance reading in presence of arabinose as the only carbon source.

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.052	0.04	0.059	0.04	0.048	0.04	0.046	0.04	0.044	0.04	0.046
B	0.041	0.059	0.041	0.057	0.041	0.048	0.039	0.046	0.041	0.044	0.039	0.047
C	0.041	0.056	0.041	0.059	0.04	0.049	0.039	0.044	0.04	0.047	0.039	0.047
D	0.04	0.059	0.04	0.062	0.041	0.049	0.041	0.046	0.039	0.049	0.04	0.044
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

Table 10. Absorbance reading in presence of raffinose as the only carbon source.

	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

5.6.2 Salt stress

Biofilm formation was studied under three conc of salt (0.6mg/ml, 0.8 mg/ml and 1mg/ml) it was observed that it decreases with increase in salt concentration (Table 11-13).

Table. 10 Absorbance reading in presence of 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. 12 Absorbance reading in presence of salt conc 0.8 mg /ml.

	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. 13 Absorbance reading in presence of salt conc 1mg/ml.

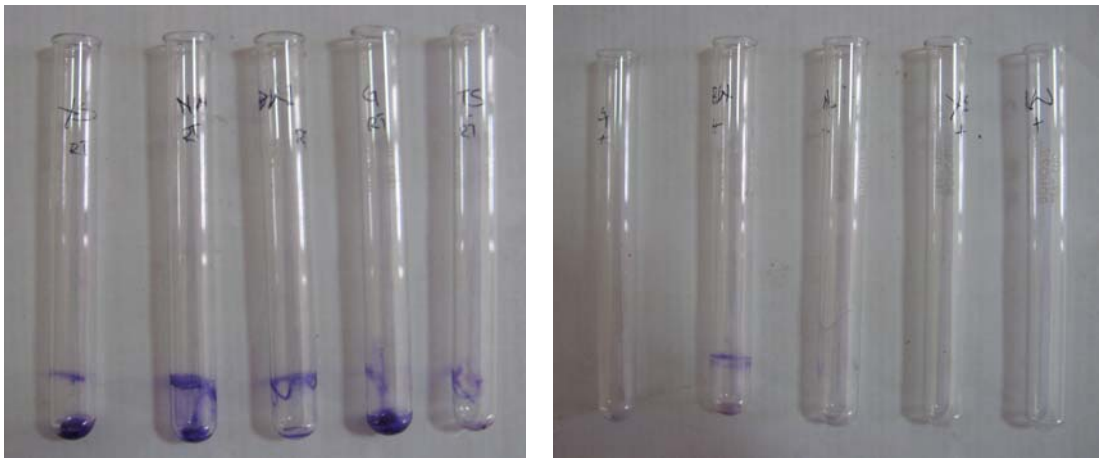
	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.042	0.041	0.044	0.04	0.044	0.04	0.049	0.04	0.052	0.041	0.053
B	0.041	0.043	0.04	0.045	0.041	0.044	0.041	0.051	0.04	0.051	0.04	0.051
C	0.041	0.043	0.041	0.043	0.041	0.043	0.041	0.05	0.042	0.051	0.04	0.052
D	0.042	0.043	0.041	0.043	0.041	0.045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

6.2.3 Temperature stress

Biofilm formation at different temperatures - room temeperature, temperature gradient and 37°C was studied and result obtained did not show any variation (Table 14-17) (Fig. 30).

Table. 14 Absorbance reading 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)**

Fig. 30 Screening for biofilm formation (a)At room temp.(b)At 37ºC

Table. 15 Absorbance reading at room temperature (Temperatue gradient).

	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.041	0.053	0.04	0.051	0.04	0.049	0.04	0.051	0.04	0.05	0.04	0.057
B	0.041	0.053	0.041	0.051	0.04	0.051	0.04	0.051	0.039	0.049	0.041	0.057
C	0.04	0.052	0.04	0.052	0.041	0.051	0.04	0.049	0.04	0.052	0.039	0.057
D	0.04	0.053	0.041	0.051	0.041	0.049	0.041	0.051	0.041	0.055	0.041	0.056
E	0.04	0.041	0.041	0.04	0.041	0.04	0.04	0.04	0.04	0.041	0.041	0.04
F	0.041	0.04	0.04	0.04	0.04	0.04	0.041	0.041	0.041	0.041	0.04	0.041
G	0.041	0.04	0.041	0.04	0.041	0.041	0.041	0.04	0.04	0.04	0.04	0.041
H	0.04	0.041	0.041	0.041	0.04	0.04	0.04	0.04	0.04	0.041	0.041	0.04

Table 16. Absorbance reading at 37ºC.

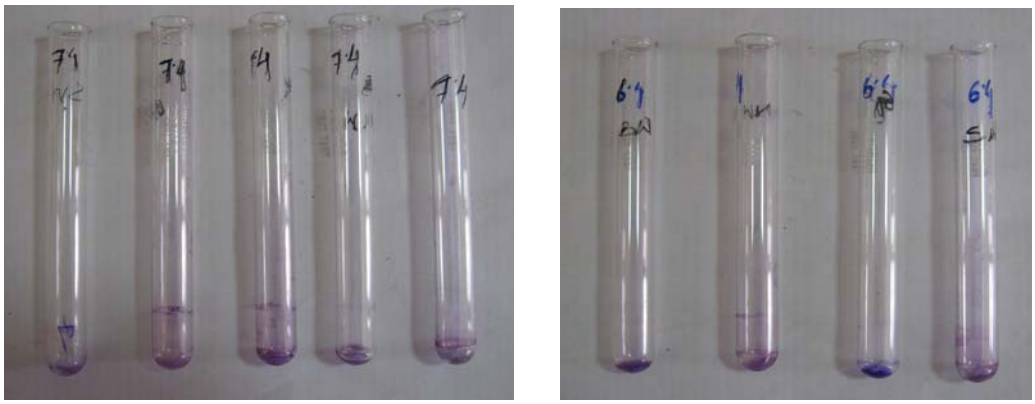
	1	2(BW)	3	4(SW)	5	6(TS)	7	8(YS)	9	10(G)	11	12(NW)
A	0.04	0.06	0.041	0.049	0.04	0.047	0.041	0.05	0.04	0.05	0.041	0.053
B	0.041	0.059	0.04	0.05	0.04	0.049	0.04	0.049	0.041	0.049	0.04	0.051
C	0.041	0.061	0.04	0.051	0.041	0.049	0.041	0.049	0.04	0.052	0.041	0.053
D	0.04	0.062	0.041	0.049	0.04	0.047	0.039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

6.4 pH Stress

Biofilm formation was studied under different pH conditions (6.4, 7.4 and 8.4). It was observed that in the range of pH 7.4 and 6.4 almost all strains showed good attachment, however, biofilm formation was poor at pH 8.4 (Table. 17-19 and Fig. 31, 32).

Table 17. Absorbance reading 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)**

Fig. 31 Screening for biofilm formation (a) At pH 7.4 (b) At pH 8.4.

Table 18. Absorbance reading 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

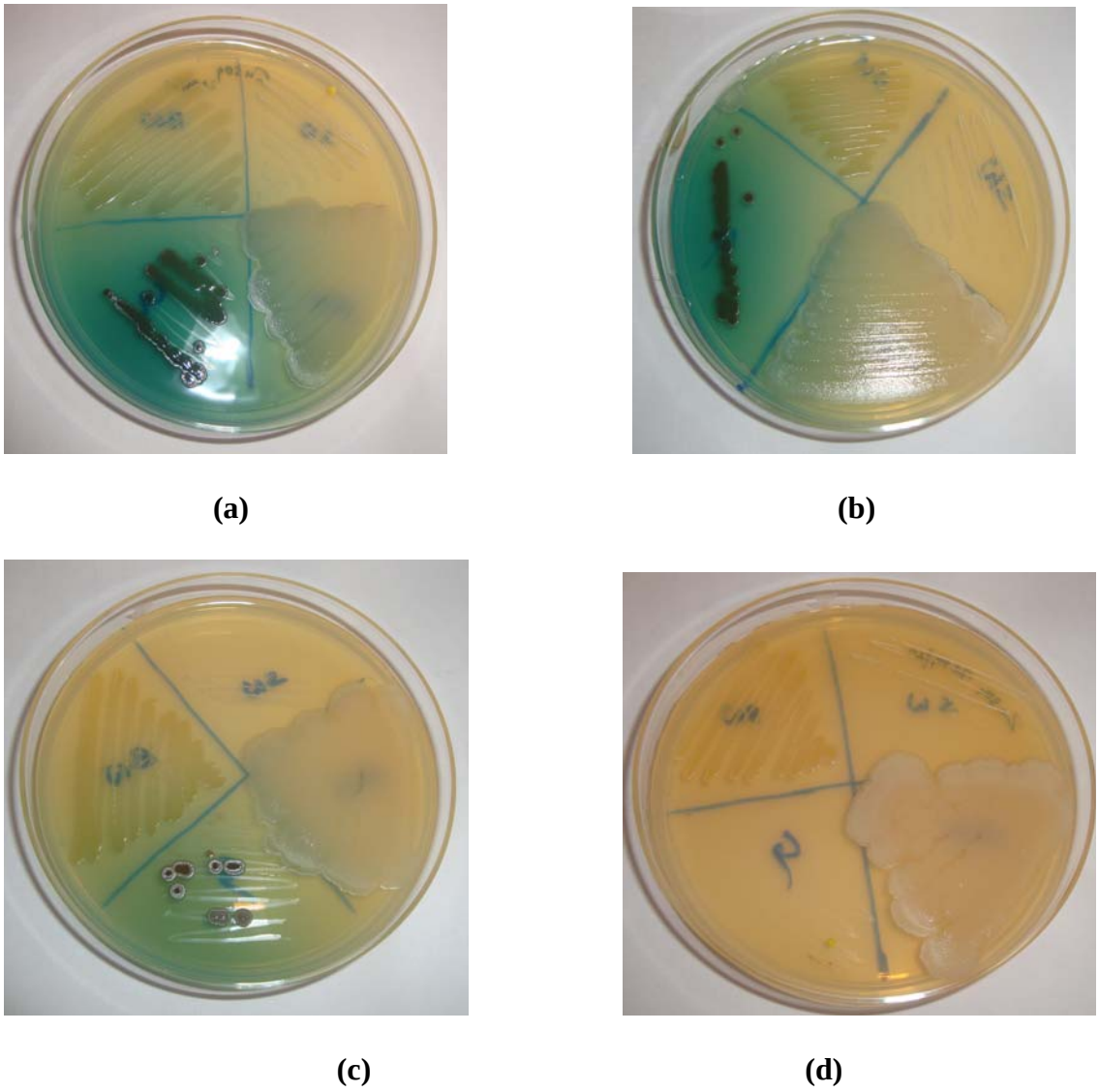
Table 19. Absorbance reading at pH 8.4.

	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

Fig. 32 Biofilm screening at pH 8.4.

6.5 Metal Stress

Biofilm formation was studied in presence of metals zinc, iron, copper, lead and cadmium. Profused 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+}), however, inhibited biofilm formation even at concentrations as low as 10ppm (Fig. 33-34).



(a) **(b)**

(c) **(d)**

Fig. 33 Plates showing 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.

Fig. 34 Tubes showing biofilm attachment in presence of 10 ppm concentration of Ca^{2+}.

5.7 Characterization of EPS Matrix

5.7.1 FTIR analysis of the extracted EPS

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. 35-38. 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.

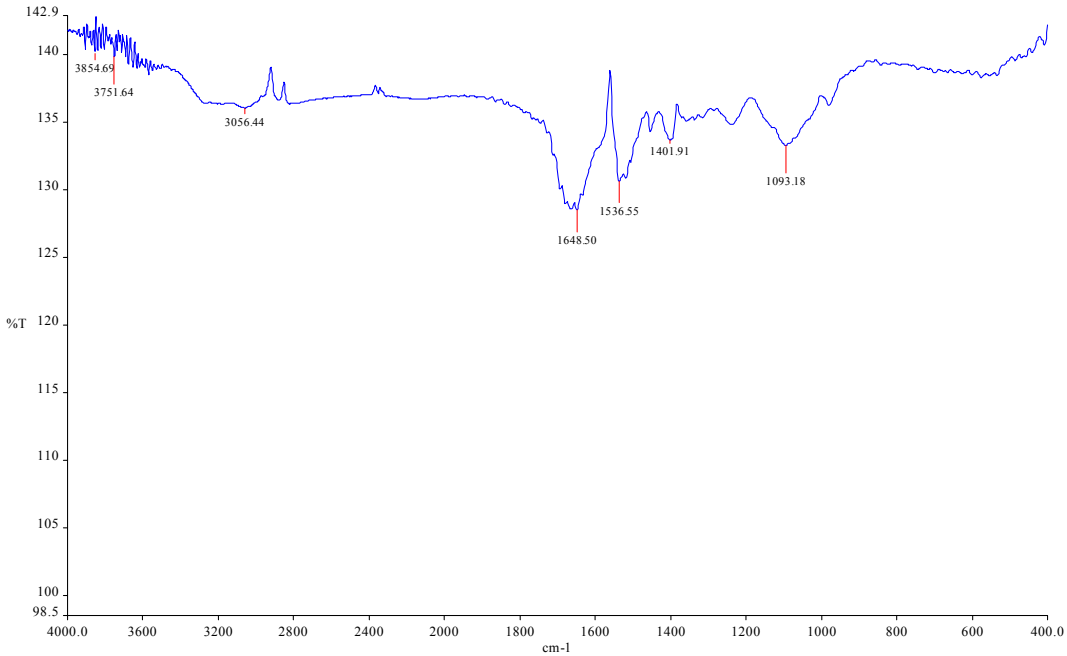


Fig. 35 FTIR analysis of EPS matrix formed by G.

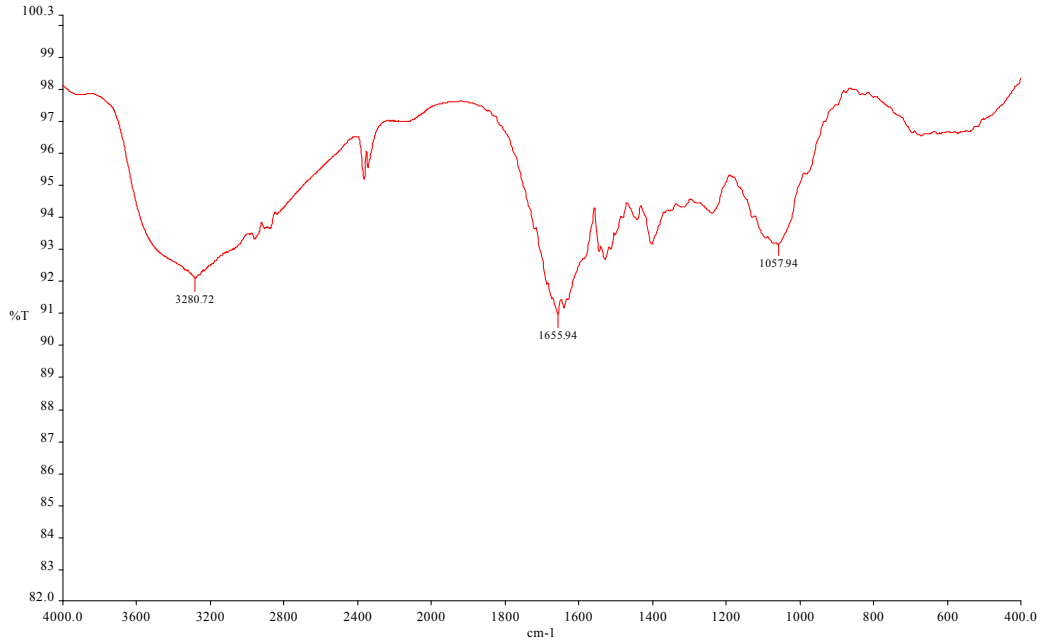


Fig. 36 FTIR analysis of EPS matrix formed by NW.

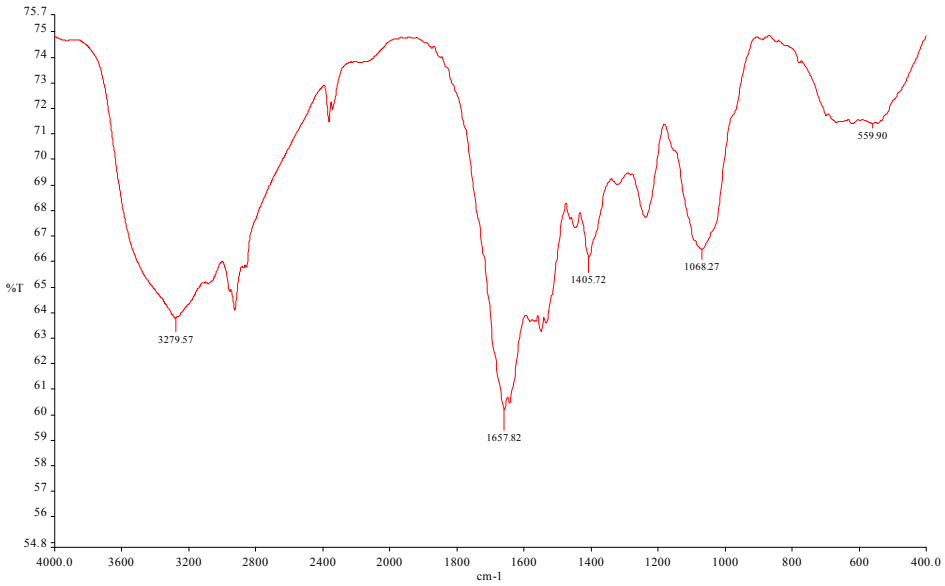


Fig. 37 FTIR analysis of EPS matrix produced by BW.

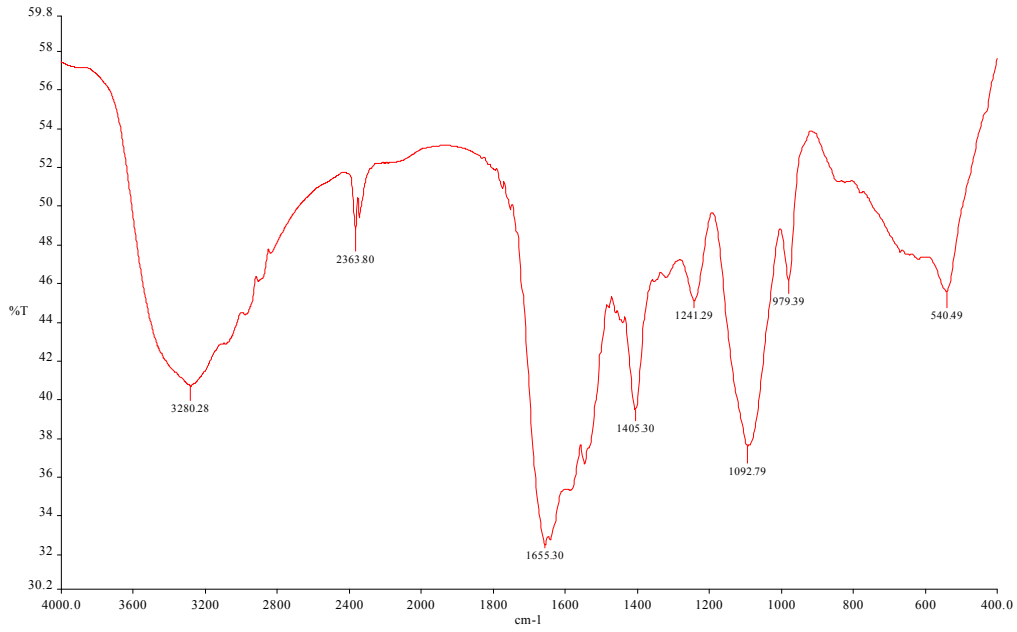


Fig. 38 FTIR analysis of EPS matrix produced by SW.

5.7.2 Chemical analysis of EPS matrix

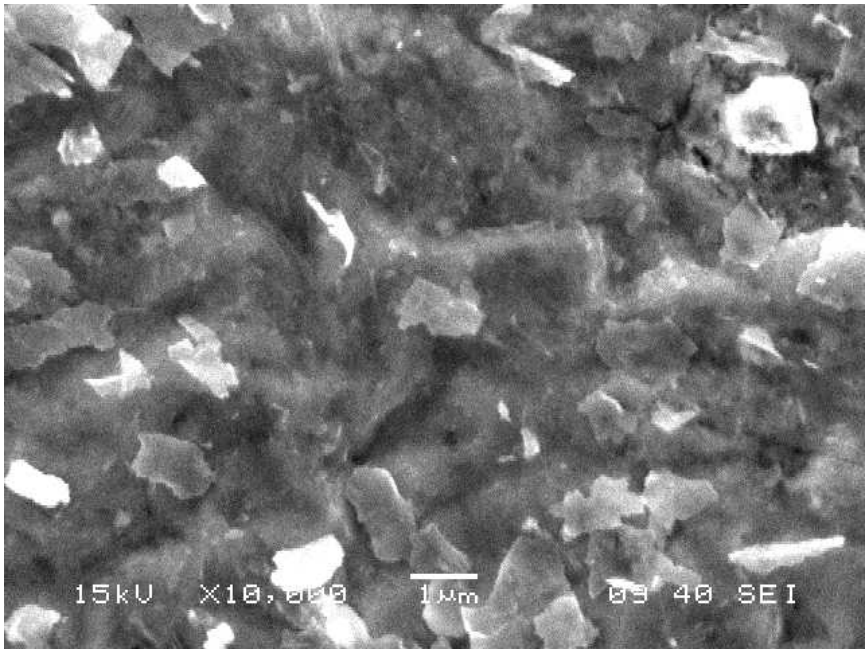
Chemical analysis of EPS matrix was done and it was observed that content of protein was more as compared to carbohydrate and it was highest in EPS extracted by NW and lowest in SW (Table. 20).

Table 20. Chemical analysis of EPS matrix.

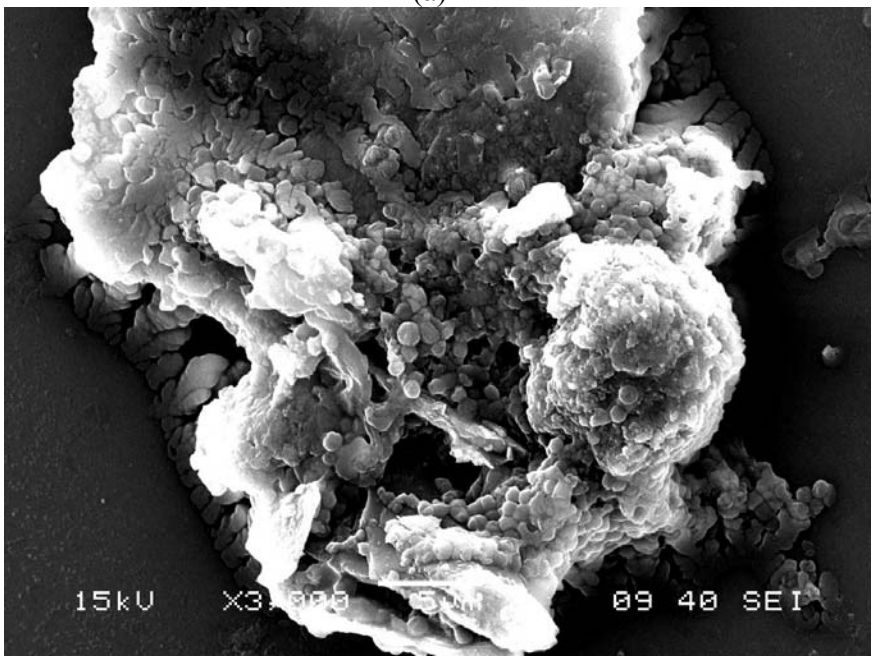
Bacterial strains	Protein content in µg/ml	Carbohydrate content in µg/ml	DNA content in µg/ml
BW	**1467**	**148.8**	**2.39**
SW	**1248**	**117.9**	**1.19**
G	**1367.5**	**154.8**	**5.11**
NW	**1698**	**105.9**	**2.48**

5.7.3 SEM analysis of Biofilm structure

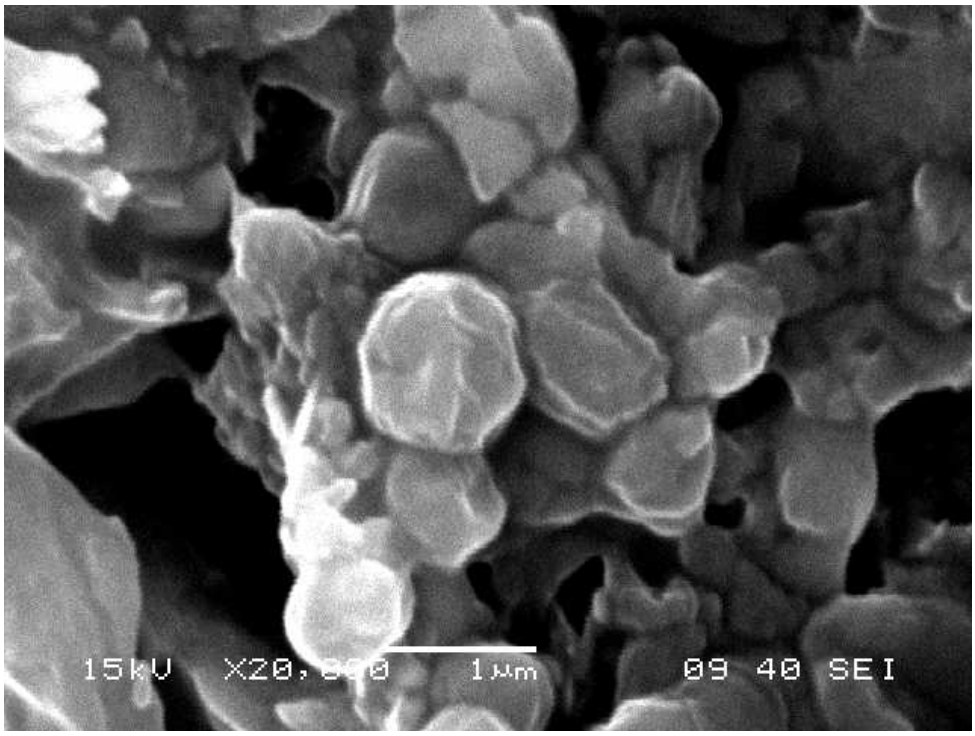
The SEM pictures for two strains NW and BW are given in Fig. 39.



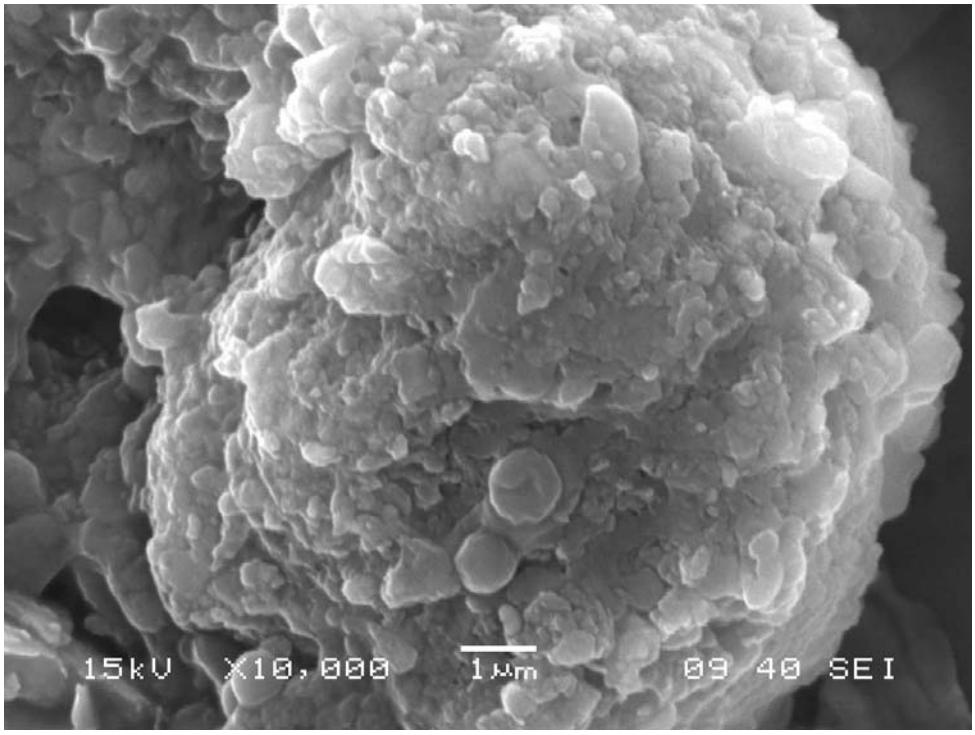
(a)



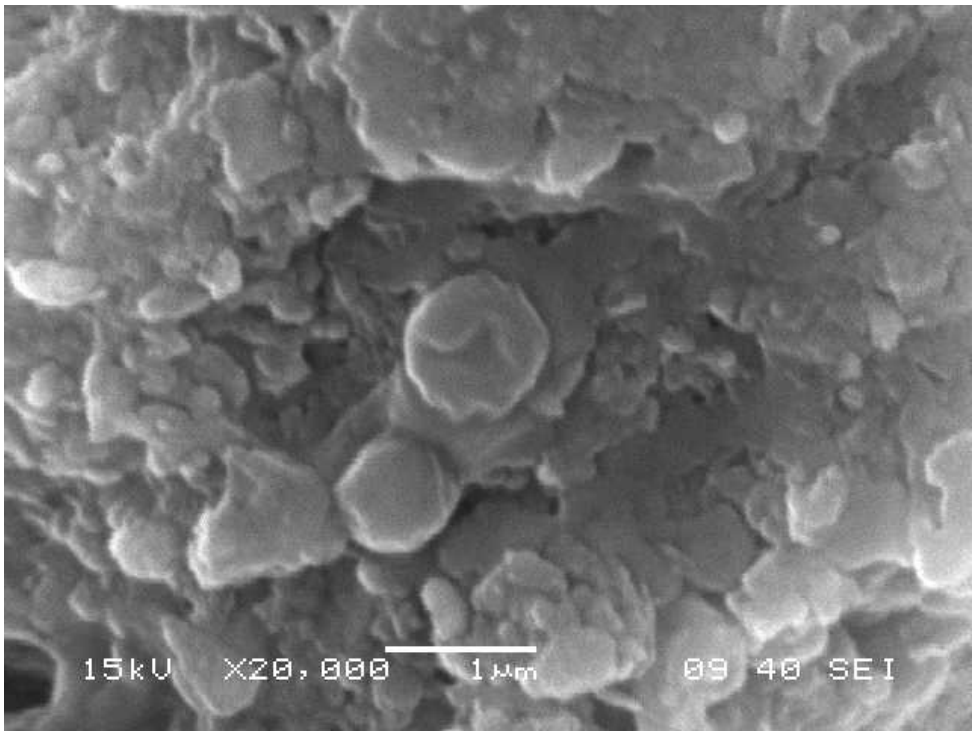
(b)



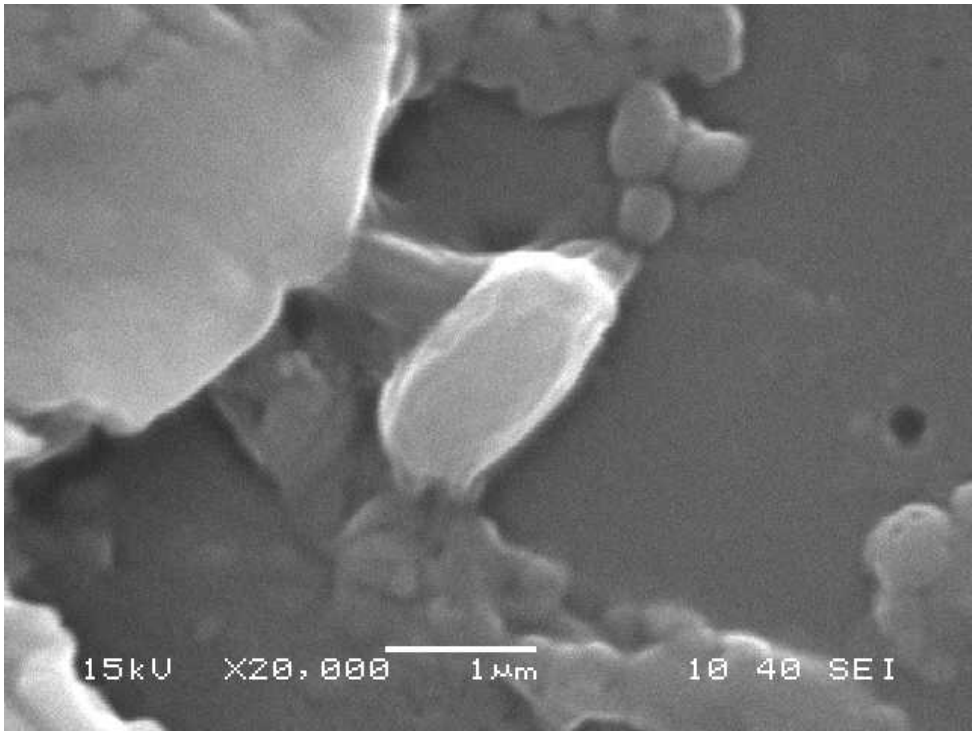
(c)



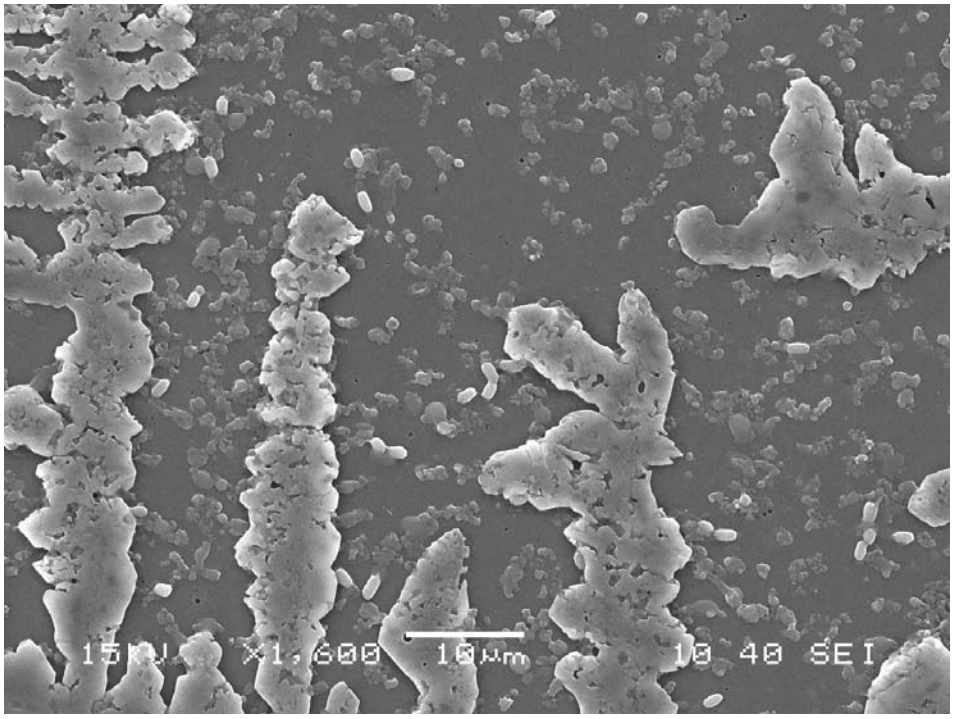
(d)



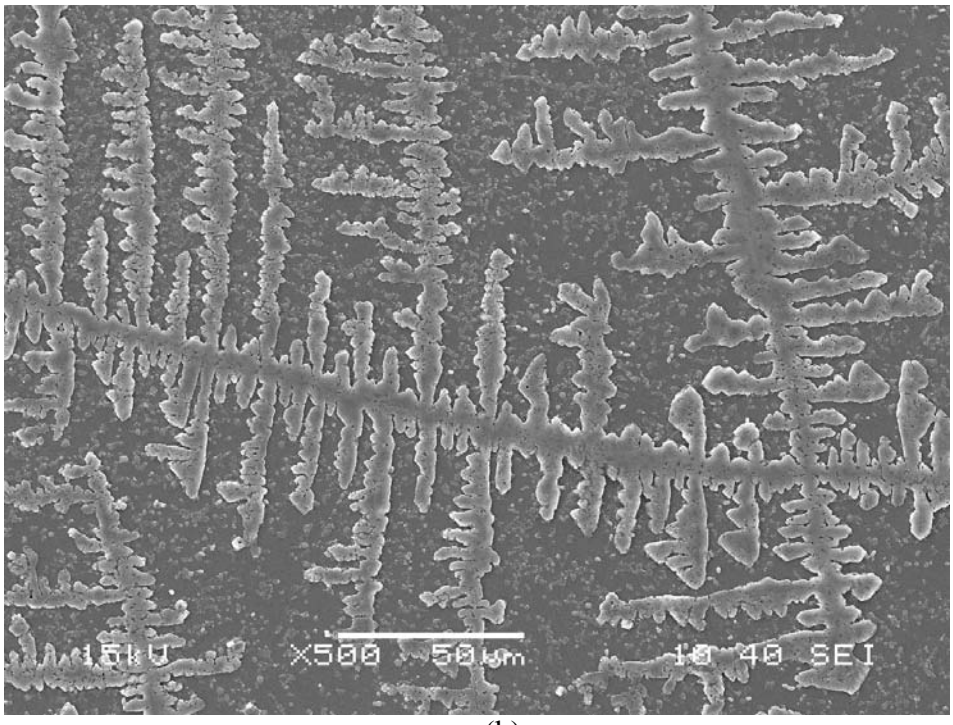
(e)



(f)



(g)



(h)

Fig. 39 SEM micrographs of biofilm formed by (a-e) by BW (f-h) NW at different magnifications.

Oral bacteria have been of late confirmed to exist as microbial biofilm. They do not exist as independent entities but function as a integrated microbial community showing concerted behaviour, i.e., the properties of which are greater than the sum of the properties of individual component species (Marsh, 2004). Development of gradient in areas of less biomass over short distances is one of the key parameters that influence microbial growth, composition and distribution. The characteristic feature of biofilm forming bacteria is production of EPS. The biosynthesis of EPS is believed to serve many functions concerning promotion of the initial attachment of cells to solid surfaces, formation and maintenance of microcolony and mature biofilm structure and enhanced biofilm resistance to environmental stress and disinfectants. In some cases, EPS matrix also enables the bacteria to capture nutrients

The composition and structure of EPS is strongly determined by the amount of fermentable sugar present. The bacterial strains studied in the present project work 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 this result is in agreement with earlier studies conducted on oral biofilm (Leme et al., 2006). Sucrose is considered the most cariogenic dietary carbohydrate, because it is fermentable, and also serves as a substrate for the synthesis of extracellular (EPS) and intracellular (IPS) polysaccharides in dental plaque (Newbrun, 1967 and Bowen, 2002) as reported earlier. However, in present study two strains (NW and G) did not utilze sucrose when it was present as the soul carbon source. There was very poor biofilm formation in case of fructose, arabinose and raffinose which is in agreement with earlier studies (Cury et al., 2000).

Test for biofilm formation in presence of metal salts of Iron and Zinc showed moderate to high inhibition of film formation. These results are in agreement with earlier studies conducted where in children in lower socioeconomic groups were fed on supplemented with Zn salts and a reduction in plaque formation was observed. The acidogenic activity of plaque in animals receiving either of the iron-sucrose meals tended to be lower. In our present study however, the inhibition of film formation in presence of

Iron salts was moderate in case of some strains while some other strains did not form films at all.

The pH and temperature of the cultured medium are also known to significantly influence biofilm formation (Czacayk and Myszka, 2007). However in present study, pH and temperature did not significantly affect biofilm formation. The results indicate that the maintenance of intracellular pH homeostasis is the basis of the enhanced physiological status and acid tolerance of biofilm cells (McNeill and Hamilton, 2004).

The chemotaxis studies carried out in present work indicates the poor response of two strains NW and G towards fructose and sucrose. This could be due to lack of fructose and sucrose utilizing machinery.

The EPS characterization of four biofilm forming strains (BW, SW, NW and G) was done and the result indicated presence of a macromolecular complex constituting of carbohydrate, protein, lipids and nucleic acids.

The present study is only a preliminary work done on isolation and characterization of oral microflora and an extensive study is needed to prevent the formation of biofilm formation in mouth. A better understanding of the significance of oral microflora as a mixed culture biofilm showing concerted behaviour will lead to efficient control strategies.

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