STUDIES ON DEPOLYMERASE ENZYMES FOR BIOFILM CONTROL

A Thesis

submitted in the partial fulfillment of the requirement for the award of the degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY

Under the guidance of:

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CERTIFICATE

This is to certify that the thesis entitled "**Studies on depolymerase enzymes for biofilm control**" submitted by Shivreet Kaur Sandhu in partial fulfilment of the requirement for the award of Degree of Masters of Science in Microbiology to Thapar University, Patiala, is a record of student's own work carried out by her. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in this thesis entitled "Studies on depolymerase enzymes for biofilm control" in partial fulfilment of the requirement for the award of the degree of Masters of science in Biotechnology, Department of Biotechnology and Environmental Sciences (DBTES), Thapar university, Patiala, is an authentic record of my work during the period of six months from January 2013 to July 2013 under the guidance of Dr. Moushumi Ghosh, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

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You never know how strong you are until being strong is your only choice…

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ABSTRACT

This study was undertaken to examine the effect of surface on biofilm formation and mainly to study the efficiency of depolymerase enzyme in biofilm control. Total 7 samples were collected from 3 different industrial sites in Patiala. A total of 200 bacterial isolates were screened for high biofilm formation and out of these, 4 were selected with maximum biofilm forming ability. The growth profile and biofilm formation were established under optimal conditions. Characterization of the biofilm revealed that extracellular polymeric substances (EPS) was comprised primarily of carbohydrates followed by proteins and a minor fraction of lipids. Biofilm formation could be established on several (PVC, glass, stainless steel) surfaces commonly used in industries. The efficiency of 3 depolymerase enzymes (proteases, lipases, α-amylases) individually and in combination was evaluated against biofilm formed by bacterial isolates. Results demonstrated that lipases exhibited a maximum reduction of 74%, followed by proteases (69.1%) and α-amylases (66.6%). Further cocktail effect of the enzymes exhibited 80% reduction in the biofilm formation. Results of this study suggest the possibility of enzymatic application for reducing biofilms produced by environmental bacterial isolates.

Keywords: Biofilm control, extracellular polymeric substances, proteases, α-amylases, lipases.

1. INTRODUCTION

Biofilms are defined as communities of microorganisms that are encased in a self-synthesized extracellular polymeric matrix (EPS) and grow attached to a biotic or abiotic surface (Sutherland *et a.l*, 2001). Biofilm formation is a sequential process, where one by one micro organisms come and adhere to the substratum (O'Toole *et al.*, 2000). The formation commences with conditioning of the surface following irreversible attachment of cells to the surface. After cell deposition, adsorption of the cells take place i.e. irreversible attachment. Cell to cell signalling starts leading to production of extracellular polymeric substances. This attachment is followed by growth into a mature complex biofilm and culminates in the dispersion of detached bacterial cells into the bulk fluid (Simoes *et al.*, 2010). Basically biofilm formation can also be termed as a continuous process and it takes days to weeks to months to form, and eventually detachment, erosion and sloughing of the biofilm occurs post maturity.

The transition of planktonic cells to sessile cells, induces the production of Extracellular polymeric substances (EPS) (Schauder *et al.*, 2008). Which constitute a matrix embedding bacterial cells having essential roles in defining the cohesiveness and other physical properties of these attached microbial colonies (Wingender *et al.*,1999). Biofilm EPSs is composed of diverse substances, including polysaccharides, proteins, lipids, nucleic acid (Nielsen *et al.*, 1996 and Tsuneda *et al*., 2003). The ability of bacteria to form biofilm have various impact on its members. In addition to increased tolerance to environment changes, resistance to antimicrobials, the biofilm bacteria shows communal existence, including division of metabolic burden and horizontal gene transfer (Kokare *et a.l*, 2008 and Jefferson *et al.*, 2004).

A major concern for many industries across the world is the formation of biofilms within their production systems. Harmful biofilms cause severe economic loss due to reduced productivity, decreased product quality, greater time, expense for biofilm removal in

industrial systems employed for wastewater management, food processing, brewing, pulp and paper manufacturing, and dairies (Lequette *et al.*, 2010). Within food industries, concern is even higher as biofilms make treating current contaminations and preventing future ones more difficult. Thus control of biofilms becomes a crucial requirement for industries to maintain product quality, safety, and efficiency. Biofilms can form in nutrient-poor environments like ultra-pure water systems also and are capable of attaching to surfaces commonly found in industrial settings, such as stainless steel (Flemming *et al.*, 1996; Simoes *et al.*, 2010).

The very nature of biofilm formation is not only detrimental but also ubiquitous. Therefore it becomes important to not only control but inhibit biofilm formation. Various methods are used to control the formation of microbial mats viz antimicrobials, disinfectants, chemicals, quorum quenching, Clean in Place method. ClP is an age old method for biofilm inhibition which is yet being practiced in industries and other various areas. Other methods include strict hygiene regimens, using resistant materials, and antibacterial coatings like paints which fight colonization (Brooks *et al.*, 2008). Therefore, focus has remained on treating or clearing them from systems. Corrosive cleaning agents and disinfectants such as chlorine, chlorine dioxide, or nitric acid or caustic sodas are commonly used. However, these are only effective against planktonic bacteria. Manual scrubbing is required to remove biofilms, and results in damage to equipment over time through corrosion (Molobela *et al.*, 2010; Cloete *et al.*, 2010). Therefore there is an urgent requirement of novel, effective and feasible alternative.

Microbial enzymes have proven to be safer and more efficient alternatives to traditional chemical means of removing biofilms. Bacterial proteases and amylases can remove biofilms completely, instead of killing only the planktonic organisms (Lequette *et al.*, 2010). This solves the major problem of biofilm materials that remain after the bacteria have been inactivated, which makes recontamination more likely. The enzyme preparation typically

prevent adhesion and remove adhered biofilms by degrading the EPS, produced by biofilms. However structurally EPS comprises of polysaccharides, proteins, lipids, nucleic acid and various cellular debris. All accomplishes various functions like providing an adhesive foundation and structural integrity. Therefore nature of enzyme becomes important for the detachment and removal of biofilm (Allison *et al.*, 2003).

In the present study three types of 'depolymerase' enzymes viz α-amylases, proteases and lipases were investigated for biofilm inhibition. The afore mentioned enzymes have been the safest for commercial use to inhibit biofilm formation in the processing unit of industries, as depolymerase poses no threat of any kind of contamination of the product with the formation of harmful by products. The enzymes can remove biofilms efficiently as they show substrate specificity, therefore targeting the structural components of the cells and inactivating the microbes leading to its lysis.

Donlan (2002) defines biofilm as an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material. Noncellular materials such as mineral crystals, corrosion particles, clay or silt particles, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix. Biofilm-associated organisms also differ from their planktonic (freely suspended) counterparts with repect to genetic expression and morphological characteristics.

2.1 Composition of Biofilm

Biofilm consists of a conglomeration of different types of biopolymers — known as extracellular polymeric substances (EPS) — that forms the scaffold for the three-dimensional architecture of the biofilm and is responsible for adhesion to surfaces and for cohesion in the biofilm. The formation of a biofilm allows a lifestyle that is entirely different from the planktonic state. EPS immobilize biofilm cells and keep them in close proximity, thus allowing for intense interactions, including cell–cell communication, and the formation of synergistic microconsortia (Flemming *et al.*, 2010). It is also known to change the physiochemical properties of the surface thereby making it easier for the cells to attach themselves to the conditioned surface. EPS not only act as a protective layer against antibiotics but also provides protection against biocides and UV irradiation. It also protects the biofilm cells under water limiting conditions by forming a protective layer and preventing dehydration (Sutherland *et al.*, 2001; Kumar,1998).

Biofilms are characterized with high levels of EPS as much as 50-90% of biofilms the total organic matter consists of EPS (Allison, 2000). Although polysaccharides dominate the EPS, exception do occur (Nielsen *et al.*, 1997). Polysaccharides and proteins are reported to account for 75- 89% of the total EPS composition (Tsuneda *et al.*, 2003), others which are

present in EPS in smaller amount, include nucleic acids (DNA and RNA), lipids and other products of cell lysis (Sara *et al.*, 2003).

2.2 Mechanism of biofilm formation

Though the exact mechanism of biofilm formation differs from bacterium to bacterium, yet the stages of biofilm development highlighted below appear to be conserved for a wide range of microbes (O'Toole *et al.*, 2000; Simoes *et al.*, 2010) (Figure 2.1).

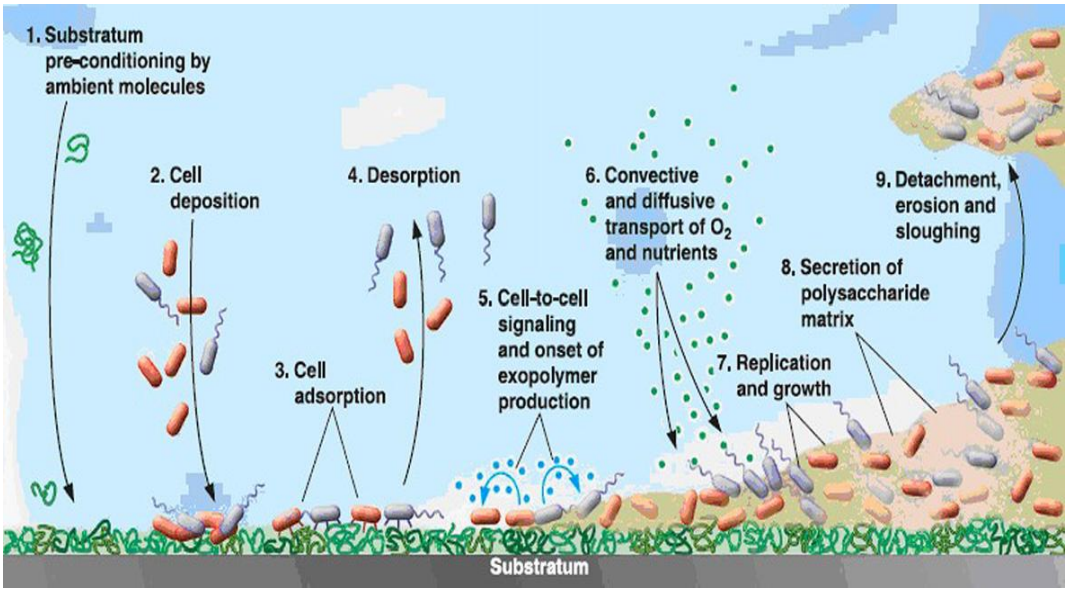


Figure 2.1. Stages involved in the process of biofilm formation (Simoes *et al.*, 2010)

The various stages in biofilm formation are as follows :

1 Formation of conditioning layer

2 Irreversible attachment

3 Biofilm maturation

4 Detachment

Biofilm formation initiates with the conditioning of the substratum. A preconditioning film is mainly composed of water, polysaccharides, lipids, proteins is known to form on the surface, resulting in nutritionally rich zone that is metabolically favourable for microbial colonization (Kumar *et al.*, 2006). Planktonic bacteria come into close approximation to the conditioned substratum and attaches itself to the substratum by electrostatic interactions, van der waals forces , specific forces or a combination if these depending on the proximity of the microbe to the matrice (Figure 2.2). The initial attachment is weak so at times many bacterial cells detach from the surface and resume a planktonic lifestyle (Watnick *et al.*, 2000). Favourable environmental conditions mediate the transition of bacterial attachment from reversible to irreversible. Irreversible attachment is further marked by production of extracellular polymeric substance. At some point following initial contact, the cells become encased in a polymeric matrix that the cells produce and thus are anchored to the substratum. The more stable attachment is due to the production by the bacteria of a complex array of extracellular polymeric substances including polysaccharides, proteins and in some cases, DNA, lipids (Allison *et al.*, 2000; O'Toole *et al.*, 1998; Finelli *et al.*, 2003).

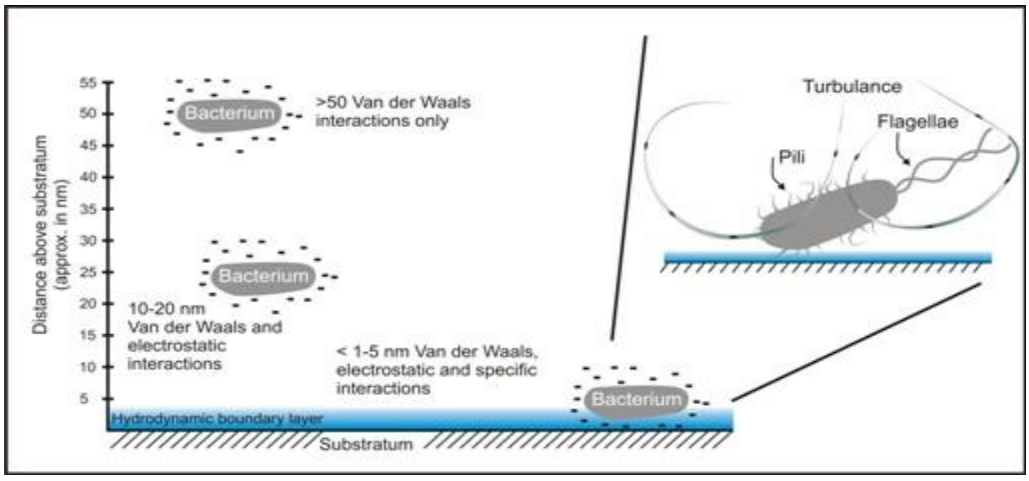


Figure 2.2 Illustrates that Van der Waals force tends to bring small objects close to the substrate wall. The electrostatic interaction plays a role in attachment due to the fact that bacterial cells and many surfaces possess a net negative electrostatic charge.

Biofilm maturation begins once when the bacteria attaches itself irreversibly to the conditioned surface. During the process the cell starts dividing, daughter cells so formed spread outward and upward from the attachment point to form macrocolonies (Nielsen *et al.*, 2000). A well developed biofilm is characterized by mushroom or pillar like structures that are interspersed with fluid like channels (Costerton *et al.*, 1995). Finally, individual cells or cell masses to separate from the substratum to which the biofilm is attached (Figure 2.3). This may include physical forces, like sloughing off, shearing and abrasion (Donlan, 2003). There may be biological reasons for biofilm detachment. It is known sudden nutrient limitation may be followed by sloughing events in which fragments of the biofilm may become detached and even the growth rate of the biofilm can influence the rate of detachment, additionally rapidly growing biofilms exhibit a greater rate of detachment than slow growing ones (Sauer *et al.*, 2008).

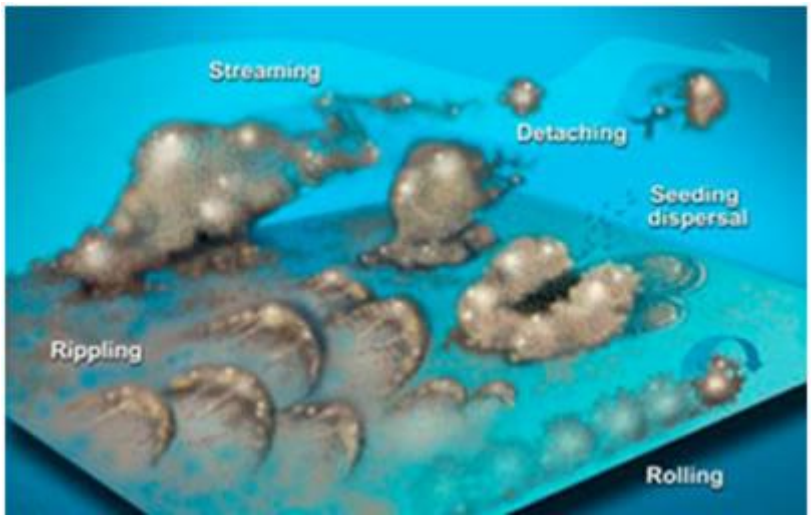


Figure 2.3 Illustrates dispersal, a phenomenon which begins in the interior of a biofilm colonies, halts as the outer layer of the colony is infringed allowing the dispersal of the cells and leaving behind a hollow, empty colony.

2.3 Factors influencing biofilm formation

Biofilm formation depends on an interaction between three main components (Table 2.1): the bacterial cells, the attachment surface i.e. substratum and the environment (Davey *et al.*, 2000; Donlan, 2002; Stoodley *et al.*, 2002).

2.3.1 Effect of bacterial cell surface

The cell wall structure is an important factor contributing to biofilm formation. The cell wall of Gram-positive bacteria consists of an inner lipid membrane surrounded by a layer of cross-linked polysaccharide referred to as the peptidoglycan. The cell wall of Gram-negative bacteria consists of a peptidoglycan comprised of acetyl amino sugars and amino acids, and an outer membrane comprised of proteins, lipopolysaccharides, and lipoproteins. Outer membrane proteins and lipopolysaccharide provide surface charge (Renner *et al.*, 2011). Other features like presence of capsule and other complex polysaccharides also aids in cell attachment to the surface. Cell surface hydrophobicity, presence of fimbriae and flagella, and production of EPS all influence the rate and extent of attachment of microbial cells (Rosenberg *et al.*, 1986). The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with an increasing nonpolar nature of one or both surfaces involved (i.e. the microbial cell surface and the substratum surface). Figure 2.4 illustrates the parameters that influence the interactions between bacteria and surfaces.

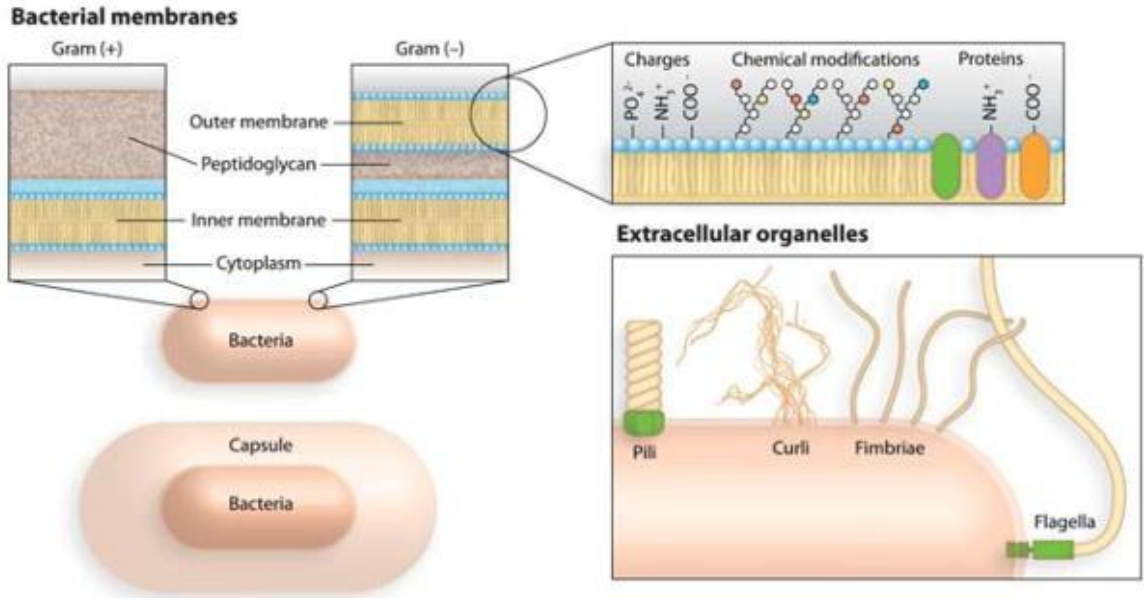


Figure2.4 Illustrates microbial surface properties influencing attachment (Renner *et al.*, 2011).

2.3.2 Effect of substratum on biofilm formation

The solid liquid interface between a surface and aqueous environment provides an ideal environment for the attachment and growth of micro-organisms. The capacity of bacterial cells to establish and form a biofilm on a given surface depends on the nature and physiochemical properties of surface. The formation of a conditioning film prior to attachment of the cells changes the physiochemical properties of the substratum which aids in attachment of the bacterial cells to the surface (Houdt *et al.*, 2010). The extent of microbial colonization increases as the surface roughness increases. This is because the surface roughness diminishes the shear force and in turn increases the surface area which facilitates the microbial adhesion. Yet another factor playing an important role in biofilm formation is hydrophobicity of the substratum (Kim *et al.*, 2000). It is directly proportional to the extent of biofilm formation. The hydrophobic interactions between the cell and the attachment surface enables the bacterium to overcome the repulsive forces and thereby attaching itself to the

substratum (Donlan, 2002). The degree of biofilm formation on different materials for various microorganism have been ranked by Rogers *et al* (1994) and by Meyer (2001) with the capacity to support biofilm growth increasing from glass, stainless steel, polypropylene ,chlorinated PVC, unplasticized PVC, mild steel, polyethylene, ethylene-propylene to latex. The suitability of plastic surfaces for bacterial attachment is due to its hydrophobic nonpolar nature with little or no surface charge, while other materials like glass, stainless steel are hydrophilic and negatively charged (Agarwal *et al.*, 2011).

2.3.3 Effect of environmental conditions

Characteristics of aqueous medium such as pH, temperature, flow velocity, ionic strength play a role in the rate of microbial attachment to a substratum (Anderson *et al.*, 2010; Donlan *et al.*, 2000; Ramli *et al.*, 2012). Biofilm formation occurs basically in stress conditions to protect the microorganisms from the adverse environment conditions. On the contrary there are studies indicating a range of nutrient conditions from scarce to excess favour biofilm formation (Prakash *et al.*, 2003). At a temperature and pH lower than the optimum for a particular species better biofilm formation occurs. Environment conditions are very subjective in nature therefore their effect on biofilm formation changes from one species to another. But for ionic strength it is found that found that an increase in concentration of several cations such as sodium, calcium, lanthanum, ferric ions affects the attachment of microbial cells by reducing the repulsive forces between the cell and surfaces (Kokare *et al.*, 2008).

2.3.4 Quorum sensing

Quorum sensing involves the production and release of molecules called autoinducers thar modulate gene expression in response to the density of a bacterial population. When autoinducers produced by one bacterium cross the membrane of another, they bind to

receptors in the cytoplasm. The autoinducer-receptor complex is then able to bind to DNA promoters and activate the transcription of quorum sensing controlled genes autoinducer molecules for gram positive bacteria are oligopeptides and that of gram negative bacteria are N-Acylhomoserine Lactones i.e. AHL (Bassler *et al.*, 2001).

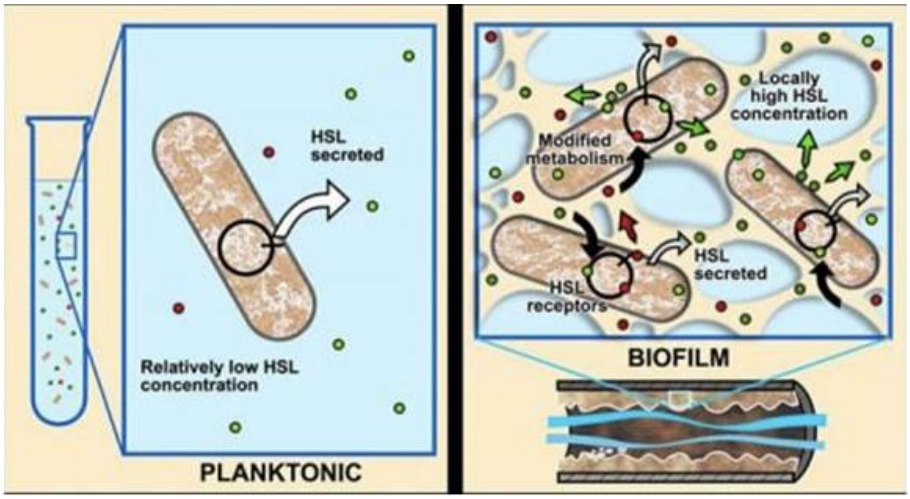


Figure 2.5 Illustrates the concept of bacterial quorum sensing. In suspension, bacterial cells produce Acylhomoserine lactone/ Homoserine lactone (HSL) signals, but the signal concentration is so low, the elevated HSL concentrations signals EPS production which helps in biofilm formation (Kjelleberg *et al.*, 2002).

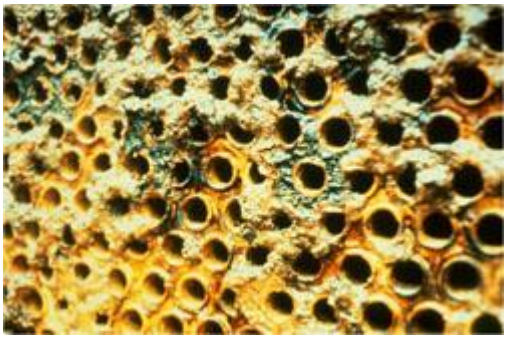
Table 2.1 Variables important in cell attachment and biofilm formation(Donlan , 2002)

Properties of the substratum	**Properties of the environment**	**Properties of the cell**
Texture or roughness	Flow velocity	Cell surface hydrophobicity
Hydrophobicity	pH	Fimbriae
Conditioning film	Temperature	Flagella
	Cations	EPS

2.4 Implications of biofilm formation

Biofilm formation is an organized continuous phenomenon where the ability to stick to surfaces and to engage in this multi step process is ubiquitous among bacteria. Biofilm formation is known to have substantial deleterious implications in various industrial processes ranging from oil drilling, paper production, food processing, ship hulls, cooling water towers, nuclear power plant circulating system, etc. Biofouling and biocorrosion cost billions every year. Industrial waste water systems are plagued by biofilm and bacterial overgrowth (Walker *et al.*, 2000). In food-processing environments, food spoilage and deterioration not only results in huge economic losses, food safety is a major priority in today's globalizing market with worldwide transportation and consumption of raw, fresh and minimally processed foods (Houdt, 2010). Biofilm formation lead to serious hygiene problems and the important aspect of controlling biofilms represent most persistent challenges with in industrial environments where microbial communities are problematic.

(a)



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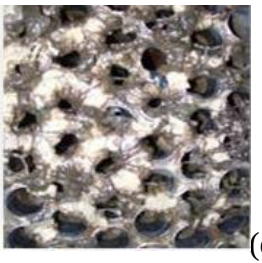


(d)

Figure 2.6 Impact of biofilm formation in Industrial units. The figure shows (a) Heat plant circulation systems; (d) Biofilm affected cooling tower of a waste water treatment plant (Lequette *et al*, 2010)exchanger of the type used in the emergency cooling water systems of nuclear power plants (b) The same type of heat exchanger heavily fouled with biofilm deposists. Such deposits can severely reduce the heat transfer capacity of heat exchangers thereby impacting the safe operation of emergency cooling water systems in power plants; (c) Biofilm in stainless steel pipe. This type of large-scale biofouling is common in many industrial water system components including, pulp and paper manufacturing, cooling towers, and nuclear power

2.5 Strategies for biofilm removal

Microbial attachment to surfaces is a fast process therefore most application are not possible to clean, disinfect frequently to avoid attachment. Apart from these the major objective is removal and control of biofilms. The deleterious effects of biofilms in various industrial unit are curbed using various methods listed :

1 Chemical disinfection

2 Mechanical treatment

3 Antimicrobial agents

4 Quorum quenching

5 Enzymatic treatment

2.5.1 Chemical Treatment

A wide range of chemical disinfectants is used in the food industry, which can be divided into different group according to their mode of action: (i) oxidising agents including chlorine-based compounds, hydrogen peroxide, ozone (ii) surface-active compounds including quaternary ammonium compounds and acid anionic compounds (Houdt *et al.*, 2010). But the biocides are not very cost effective and also certain microbes are known to produce the enzyme catalase which is responsible for degrading H_2O_2. Ozone which is another oxidising biocide is much stronger but it has been limited due its low water solubility, especially at high temperature. Also ozone requires production at the industrial unit itself as its of a very unstable nature. Mostly the action of these biocides turns to be corrosive which basically limits the use of these chemicals (Keevil *et al.*, 1999; Charaklis *et al.*, 1990). The increased resistance of biofilm cells to biocides, which is at least partially because of interference of the

exopolymeric matrix, explains why the disinfectant most effective to planktonic cells is not necessarily the most active against biofilm cells. Chemical disinfectants can also enhance the biodegradable substances. These substances can be used by the bacterial cells as energy sources and promotes biofilm formation.

2.5.2 Mechanical Treatment

Mechanical treatment methods have been studied as a alternative to chemical methods. Most important among these as reported by Jessen for removing biofilm is Clean In Place (CIP) method. The mechanical method includes the physical methods like mechanical forces, application of heat, mechanical scrubber, pressure cleaners, etc. Manual scrubbing required to remove biofilms, results in damage to equipment over time through corrosion (Molobela *et al*., 2009). It is also expensive as unwanted halt of the system involves shutting down of the unit for that particular period of time. Yet another demerit is that it is time consuming. It is also impractical for larger machines where the more affected regions like joints, filters, or gaskets are not easily accessible. All these inaccessible parts are the most favoured places for biofilm formation therefore limiting the use of CIP method.

2.5.3 Antimicrobial agents

Antimicrobial agents are compounds that inhibit or kill microorganism. They are either bacteriostatic or bactericidal. Different antibiotics have different modes of action, owing to the nature of their structure and degree of affinity to certain target sites within bacterial cells: inhibition of the cell wall synthesis, inhibition of ribosome function, inhibition of nucleic acid synthesis, inhibition of cell membrane function. It is very well known that biofilms are resistance to antibiotics due to the presence of EPS (Sutherland *et al.*, 2010). On using antibiotics certain biofilm cells survive and this resistant fraction goes on to become the persistors. The persistors then form a resistant matured biofilm. Figure 2.7illustrates drug

resistance in Biofilm. The EPS is represented in yellow and bacteria as blue ovals. Biofilms are marked by their heterogeneity and this includes gradients of nutrients, waste products and oxygen. Mechanism of resistance in the biofilms include increased cell density and physical exclusion of the antibiotic, following changes can occur in biofilm-growth bacteria (Mah *et al.*, 2001): induction of general stress response, increasing expression of multiple drug resistance (MDR) pumps, activating quorum sensing systems, and changing profiles of outer membrane proteins (OMP).

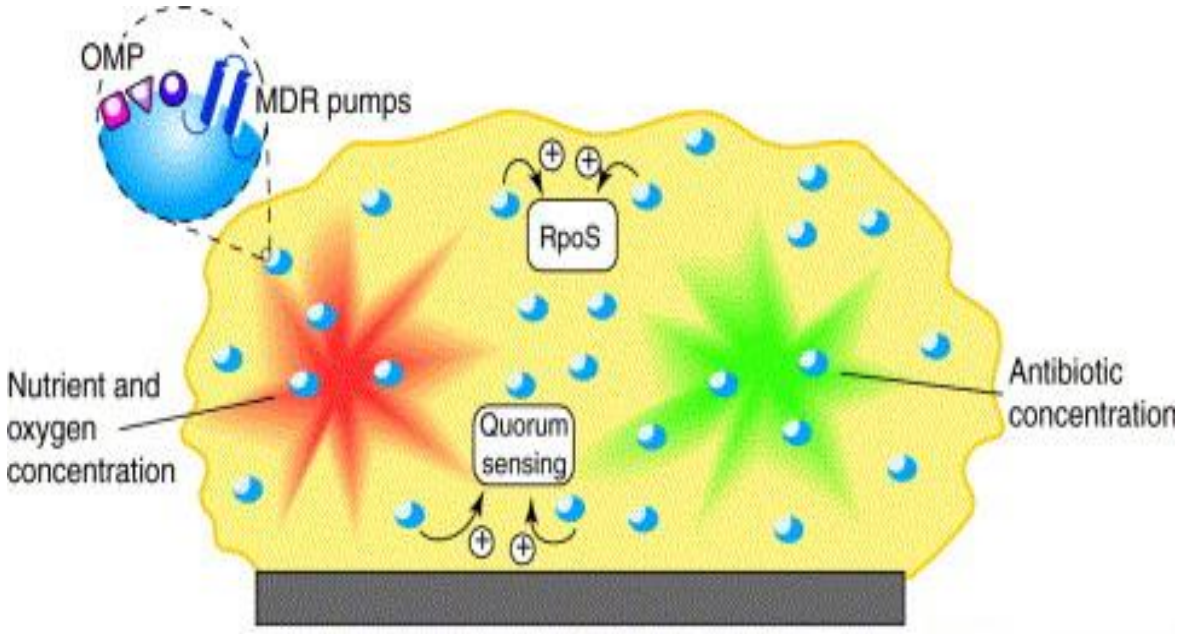


Figure 2.7 depicts a schematic of mechanisms that can contribute to the resistance of biofilm grown bacteria to antimicrobial agents.

2.5.4 Quorum quenching

Microbial cells rely on quorum sensing to activate pathogenic factors and formation of biofilms. Any compound that prevents production of signal molecules or interactions between signal molecules and cognate receptor proteins might block bacterial quorum sensing and its gene expression. The discovery of bacterial quorum sensing mechanisms has led to identification of some compounds or enzymes that quench quorum sensing, called QS

interference. Evidence has accumulated that such QS interference can be developed as promising approaches to control biofilm formation and microbial infections. Though quorum quenching is reliable and effective but in an industrial set up not really feasible. Any compound that prevents production of signal molecules or interactions between signal molecules and cognate receptor proteins might block bacterial quorum sensing and its gene expression (Turner *et al*, 2002; Tian *et al.*, 2012). Figure 2.8 depicts the Quorum sensing inhibition

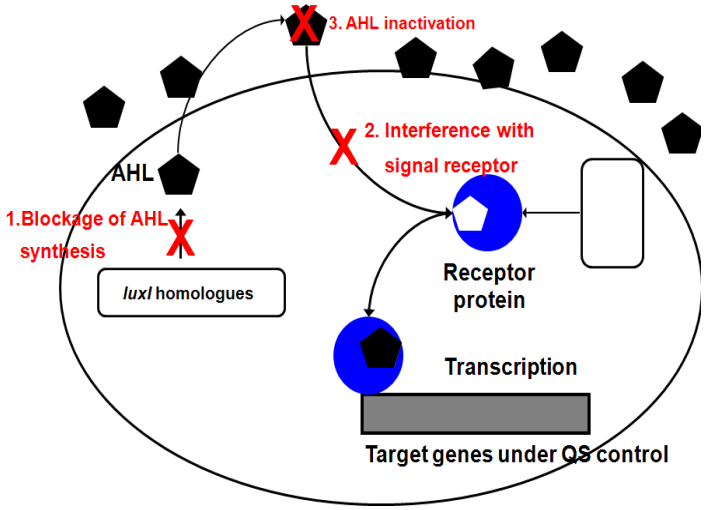


Figure2.8 illustrates strategies of quorum sensing mechanism. It is made possible by AHL inactivation, blocking of AHL synthesis or by interfering with signal receptor.

2.5.5 Enzymatic treatment

Effective control strategies should be there to prevent the formation of biofilm, therefore a method for inhibiting both cell to cell and cell to surface association of bacterium is required. Enzymes have an aptitude to detach preexisting aggregates and biofilms. Enzymes, specifically the group of depolymerases, are an attractive option because they are readily available, as some are already produced at an industrial scale, biodegradable, and generally

have a lower toxicity than chemical and enzyme alternatives such as oxidases (Leroy *et al.*, 2008). One of the reason for their attractiveness is that the use of enzyme treatments to breakdown EPS in biofilms is a possible alternative when standard cleaning agents do not give satisfactory results in removing biofilms. Depolymerase enzymes are able to cleave the proteins at specific amide bonds, also cleave the carbohydrate and lipid moiety effectively destroying the physical structure of a biofilm. Instead of only killing the majority of planktonic bacteria and some of the sessile bacteria, they break up the biofilm and allow its removal from the system altogether (Xavier *et al.*,2005). Thus they help to eliminate existing biofilms and prevent bacteria from adhering to surfaces thus making them a desirable option (Johansen *et al.*, 2000). Infact use of depolymerases as a means to remove biofilms has been investigated in several recent studies using proteases and amylases (Johansen *et al.*, 1997; Orgaz *et al.*, 2006; Molobela *et al*, 2010). Due to substrate specificity of enzymes and the heterogeneity of the EPS, the removal of biofilms has shown to be more effective when enzymes were used in combination or a cocktail of enzymes(Meyer *et al.*, 2003) were used along with detergents (Johansen *et al.*, 1997).

3. SCOPE

The methods used in the industries to tackle the biofilm formation have limitations. For instance in CIP method, success in removing biofilm formed in the joints of the pipelines is less. Another problem with CIP is that it clears the existing biofilm but does not avert its formation. Chemicals are still being used because of cost considerations but the resistance of biofilms to antibiotics, and even to oxidizing biocides has been well documented. The treatments so far used for removal of biofilm lack specificity in their action.

Recent studies have demonstrated the efficiency of enzymes. They are not only more efficient they will also help in running industrial units with incurring less economic losses. The presence of biomolecules (sugars, proteins, lipids, etc) in the EPS in varying proportions among micro-organisms, therefore making it crucial to study the effect of these enzymes in biofilm control.

In the present study, attempt has been made to characterize biofilm forming bacteria and check the efficiency of enzymes in biofilm inhibition. Therefore the following objectives were proposed :-

- Isolation of biofilm producing micro organisms from diverse sources, screening and characterization of biofilms.
- Simulation studies of biofilm formation on various matrices.
- The effect of ‘depolymerase’ enzymes on biofilm inhibition

4.1 Sample collection

The sludge and soil samples were collected from various industry, domestic, river sites listed in the Table 4.1 from Patiala to isolate the biofilm producing bacteria. The samples were collected using a sterile spatula and stored in sterile zip locker bags (Hi media, Mumbai). All samples were transported on ice and analysed within 4-8 hours of receipt.

SITE	SOURCE
Federal Mogul Goetze (India) ltd	Fresh soil sludge
Federal Mogul Goetze (India) ltd	Fresh soil sludge
Federal Mogul Goetze (India) ltd	Fresh soil sludge STP
Federal Mogul Goetze (India) ltd	Wet soil
Domestic sewage	Sludge
River I, Patiala	River side soil
River II, Patiala	River side soil

4.2 Reagents and chemicals

All the chemicals and reagents used for microbiological and chemical determinations were purchased from Sigma. Standard media components were purchased from Fisher Scientific (USA) and Hi-media (Mumbai, India). Media solutions were routinely sterilized by autoclaving at 121°C and 15 psi for 15 min and were allowed to cool below 50°C before use. Nutrient broth (13 % w/v) was used for growth of microorganisms. The screening medium used was 'Luria Broth' was used (Viktoria *et al.*, 2011). The enzymes used were purchased from Hi-media (Mumbai, India).

4.3 Isolation and screening of biofilm producing bacteria

The various samples collected were weighed and 1 gm of the sample was dissolved in 50 ml distilled water. The solution so formed was subjected to vigorous shaking and left undisturbed for a 10 minutes, following a serial dilution upto 10^{-5} were made in saline. From the dilutions 10^{-3}–10^{-5} 100 µl were plated on biofilm nutrient agar medium and the plates were incubated at 37°C for 24 to 48 hours. Out of 200 discrete colonies, 22 were selected based on their mucoid colony morphology. The isolates were repeatedly streaked and purified by streak plate technique. Glycerol stocks were prepared of the isolates and were kept for preservation at -80°C.

The screening of biofilm producing isolates was based on the rapid crystal violet assay. Biofilm production was estimated using a modified method of Judith *et al.*, (2011), a semi quantitative method or crystal violet assay, 300 µl of standardized bacterial suspension in exponential phase was revived in biofilm inducing media for the 22 isolates were dispensed in the wells of 96-well flat bottomed microtiter plate and kept for incubated at 37°C for 48 hours. The control well contained only the biofilm inducing media devoid of any culture. Following incubation, the unbound cells were removed by inverting the microtiter plate and vigorous tapping. The wells were then rinsed with phosphate buffer (pH 7.2). Three hundred µl of 1% (w/v) crystal violet solution was then added to the wells for 5 minutes. The wells were then washed with deionized water extensively and plates were allowed to dry. Destaining was done with 300 µl of 30% (v/v) acetic acid. The absorption of the eluted stain bound to the biofilm was finally measured at 595 nm. The strains showing the maximum absorption were selected for further analysis (Djordjevic *et al.*, 2002)

4.4 Simulation studies on various matrices

Simulation studies of biofilm formation was done on various matrices viz. glass, stainless steel and polyvinyl chloride (PVC) according to the modified method of Jung *et al*., (2002) and Rogers *et al.,* (1994). The glass, stainless steel and PVC were washed with distilled water, wiped with ethanol, later all three were autoclaved. The sterile matrices were then placed into the medium containing 1% inoculum. The biofilm formation on the three matrices were periodically observed after every 12 hrs and over the span of 72 hrs. After removing the glass, steel and PVC they were washed gently with sterile distilled water and left for drying. Later the matrices were subjected to staining with 0.1% crystal violet (w/v) for 10 minutes. Following staining the matrices were washed again with distilled water and the biofilm so formed was observed qualitatively.

4.5 Identification of the isolates

All the isolates were morphologically and biochemically characterized according to Bergey's Manual of Determinative Bacteriology (Buchanan *et al., 1974*) and are briefly discussed below.

4.5.1 Morphological identification

The cell morphology was done using Gram staining. The diluted suspensions of the bacteria were smeared on clean slides, air dried and heat fixed. 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 oil immersion. Cells were

then identified by the colour observed purple for Gram positive and pink or red for Gram negative cells. Motility is used to check the ability of bacteria to migrate away from the line of inoculation. The bacteria was inoculated into SIM (Sulfide, Indole, Motility) agar tubes by stab inoculation method using a sterile inoculating needle and was observed after 24-48 hours incubation. If the test organism migrates away from the line of inoculation, then the bacteria is motile. Colony morphology was observed in overnight plate cultures on Luria Agar.

4.5.2 Biochemical Identification

A. Methyl Red Test

Some bacteria perform mixed acid fermentation. The by-products are mixtures of large amounts of stable acids. Other fermentative organisms produce smaller amounts of less stable acids. The Methyl-Red test tests for the ability to perform mixed-acid fermentation. MR-VP broth contains glucose, peptone, and a phosphate buffer. Organisms that perform mixed-acid fermentation produce enough acid to overcome the buffering capacity of the broth, so a decrease in pH results. Organisms that perform other kinds of fermentation cannot overcome the buffering capacity of the broth. After incubation, the pH indicator Methyl Red is added to the broth. Methyl Red is red at pH below 4.4 (this would be a positive result) and yellow at pH above 6.0. An orange color indicates an intermediate pH and would be considered a negative result. MR-VP broth was inoculated with a 1% inoculum of 24 h overnight grown culture. The inoculated tubes are kept for 24 - 48 h , following incubation few drops of methyl red indicator was added. Red colour indicates a positive result, yellow shows a negative result.

B. Voges-Proskauer Test

VP tests are used to determine what are the end products when the test organism degrades glucose that is present in the MR-VP broth. MR-VP broth was inoculated with a 1% inoculum of 24 h overnight grown culture. The inoculated tubes are kept for 24- 48 h. The reagents used for the VP test are Barritt's A (α-napthol) and Barritt's B (potassium hydroxide). When these reagents are added to a broth in which acetyl methyl carbinol is present, they turn a pink-burgundy color (a positive VP test).

C. Citrate utilization test

The citrate test utilizes Simmon's citrate media to determine if a bacterium can grow utilizing citrate as its sole carbon and energy source. Simmon's media contains bromothymol blue, a pH indicator with a range of 6.0 to 7.6. Bromthymol blue is yellow at acidic pH (around 6), and gradually changes to blue at more alkaline pH's (around 7.6). Uninoculated Simmon's citrate agar has a pH of 6.9, so it is an intermediate green colour. Growth of bacteria in the media leads to development of a Prussian blue colour (positive citrate). On Simmon Citrate agar slants a 24 hrs old culture is streaked using sterile technique. The tubes were then incubated at 37°C.

D. Indole Production Test

An essential amino acid Tryptophan can undergo oxidation by enzymatic reaction of certain bacteria. Few bacteria hydrolyze tryptophan with the production of indole. The presence of indole was detected by addition of Kovac’s reagent, which produces cherry red reagent layer. Using sterile techniques SIM agar tubes were inoculated with 24-48 hrs grown cultures with stab inoculation and incubated at 37ºC for 24 hrs. Cultures producing a red layer on the

addition of Kovac's reagent are indole positive. Kovac's reagent is composed of *p*-dimethylaminobenzaldehyde, butanol and hydrochloric acid.

E. Starch hydrolysis test

The purpose is to see if the microbe can use starch, a complex carbohydrate made from glucose, as a source of carbon and energy for growth. Use of starch is accomplished by an enzyme called alpha-amylase. A medium containing starch is used. After inoculation and overnight incubation, iodine reagent is added to detect the presence of starch. Iodine reagent complexes with starch to form a blue-black colour in the culture medium. Clear halos surrounding colonies is indicative of their ability to digest the starch in the medium due to the presence of α-amylase. Starch agar plates were made and divided into smaller sections. Spot inoculation method was used for inoculation and the plates were incubated at 37 ºC for 24-48 hrs. Following incubation the plates were flooded with Gram's iodine solution and allowed it react for 30 sec and the excess was drained out. Then the cultures were observed for the presence and absence of the blue-black colour surrounding the growth of each colony.

F. Catalase Test

Trypticase soy agar plates were inoculated by spot inoculation technique. The plates were incubated for 24-48 hrs at 37°C. The next day 3-4 drops of hydrogen peroxide (3%) were allowed to flow over each colony. Cultures were observed for the presence or absence of bubble formation.

G. Hydrogen Sulfide Test

Hydrogen sulfide test is used to detect whether microorganisms are capable of producing hydrogen-sulfide from substances such as sulphur containing amino acids or inorganic sulfur compounds. SIM agar tubes were inoculated with 24-48 hrs grown cultures with stab

inoculation and incubated at 37ºC for 24 hrs. The strains that were produce black coloration along the line of the stab inoculation were positive for Hydrogen Sulfide test. Uninoculated tube was taken as negative control.

H. Urease Test

Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end product ammonia. The presence of urease is detectable when the organisms are grown in urea broth medium containing the pH indicator phenol red. As the substrate urea split into into products, the presence of ammonia creates an alkaline environment that causes the phenol red to turn to a deep pink. Urea broth was inoculated with 24 hrs grown cultures using loop inoculation and incubated at 37ºC for 24 hrs. On the addition of phenol red indicator the strain that produces pink colour was positive and no change in the colour of the broth was negative for urease test.

4.5.3 Molecular identification

All the molecular biology techniques were performed as outlined in Sambrook *et al*., (1998) unless noted. DNA modifying enzymes were obtained from Promega (Fisher Scientific, USA). PCR reactions were conducted using universal primers P0 and P6. The PCR mixture (total volume 100 µl) contained 1 µl *Taq* (10 X) commercial buffer, 5 µl purified DNA (50-100 ng), 150 µM of each dNTP, 500 ng of each primer and 2.5 U *Taq* polymerase. The program for 16S rDNA was as follows: The reaction mixtures were first incubated for 5 min at 95°C and then cycled for 36 cycles according to the following temperature profiles: 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, followed by final extension for 10 min at 72°C, unless otherwise specified, on a thermal cycler. The resultant amplicon was sent for the sequencing. (Sambrook *et al*., 1998)

4.6 Biofilm production assay

Biofilm optical density, measured as intensity reduction of a light beam transmitted through the biofilm, correlates with biofilm mass. Absorbance, which is reduction in intensity of light is proportional to the bacterial cells in medium (Beer-Lambert Law); also in biofilm kinetics the absorbance is proportional to the attached cells along with the EPS (Bakke *et al.*, 2001). Therefore a semi quantitative microtiter plate assay (polystyrene, 96-well) was used for biofilm production assay and growth kinetics.

4.6.1 Growth conditions and biofilm formation

To observe the growth kinetics of isolates the media was inoculated with a 1% inoculum (O.D. 0.3) in 250mL sterile erlenmeyer flask, incubated at 37°C with shaking. Samples were asceptically withdrawn after every 4 hrs and centrifuged at 8000 rpm for 5 min. Pellet was washed and resuspended in saline and absorbance was noted at 600 nm. A graph of time vs absorbance was plotted to determine the planktonic growth kinetics of isolated strains. Biofilm formation was tested in 96-well polystyrene flat bottom plates, 200µl of cell suspension was transferred to each well of the plate and incubated at 37°C. Test medium without cells was added to the wells and used as negative control. After the biofilm formation the unbound cells were removed by inverting the microtiter plate and vigorous tapping. The wells were then rinsed with phosphate buffer (pH 7.2). Adhered cells were stained with 200µl of 1% (w/v) crystal violet solution was then added to the wells for 5 minutes. The wells were then washed with deionized water extensively. The plates were then allowed to dry. Following washing, 200µl of 30% (v/v) acetic acid was added as the destaining agent to each well for 15 minutes. 100µl of solution was taken and transferred to fresh microtiter plate after brief mixing. The absorption of the eluted stain was measured at 595 nm.

4.6.2 Biofilm quantification by crystal violet staining

The absorbance values of negative controls (containing no cells) were substracted from values of test wells to minimize background interferences. Biofilm production quantities were reported as arithmetic mean ± standard deviation (SD) of O.D. (595 nm) values of three replicates. The OD values were plotted against time interval to determine biofilm formation kinetics of the isolates.

4.7 Characterization of biofilm of the selected isolates

4.7.1 Nature of biofilm

The characterization was based on alcian blue test (Passow *et al.*,1995) which indicates the presence of acidic polysaccharides , sudan black which is a lipophylic dye (Liu *et al.*, 1998) , calcoflour assay where this binding agent binds with carbohydrate showing fluorescence (Woods *et al.*, 1980) and also the well known crystal violet assay (Burton *et al.*,2007).

A. Alcian Blue Test

The presence of acidic polysaccharides was detected using the Alcian blue binding Test (Bobber, 2005). Alcian blue a cationic dye has been used to measure biofilm EPS through its ability to form insoluble precipitate after binding to the half ester sulfate and carboxyl functional groups of the biofilm EPS. One hundred micro litres of supernatant of centrifuged culture at 10000 rpm for 5 minutes at 4°C was taken, to which 700 µl of 0.5M acetic acid was added. Thereafter 200 µl of alcian blue dye was added. After incubation of the solution for 2 hours at room temperature it was centrifuged at 8000 rpm for 5 minutes at 4°C. The absorbance was taken at 580 nm , taking distilled water in place of culture as blank.

B. Sudan black assay

Sudan black B dye binds to the lipid content and imparts a bluish black colour to the colony. The isolates were grown as single colonies on plates containing nutrient broth and were allowed to grow for 24-48 hrs at 37°C. After incubation the plates were flooded with 0.02% Sudan black solution and kept undisturbed for 10 min. The plates decanted and rinsed with 10 ml of 100% ethanol, positive results showed a black coloured colony while a negative result showed a clear zone or no colouration (Liu *et al.,* 1998). Sudan black a lipophylic dye has been used to detect the presence of lipid. It is thought that the staining of lipids depends on the physical properties of solution or adsorption. When the Sudan Black stain is dissolved in organic solvent, it exhibits a greater solubility, therefore during staining, dyes will migrate into lipids from organic solvents resulting in lipid staining.

C. Calcoflour assay

The presence of β(1,3) and β(1,4) linkages between carbohydrates can be detected using this fluorochrome binding agent calcofluor (Christine *et al.*, 2010). For calcoflour binding assay the plates were streaked with the selected isolates on nutrient agar plates containing calcoflour (200µg/ml). The plates were then incubated for 24-48 hrs at 37°C. After incubation the plates were observed under UV transilluminator to check for presence or absence of fluorescence (Woods *et al.,* 1980).

4.7.2 Compositional analysis

(I) Extraction and Purification of Biofilm EPS

The selected strains were inoculated in Luria broth and incubated for 48 hrs at 37°C. the culture was centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant was extracted, double volume of chilled ethanol was added to the supernatant and incubated at 4°C

overnight. To extract the biofilm, the solution was again centrifuged at 12000 rpm for 10 min at 4°C. The pellet was then washed with deionized water, dissolved in 3-4ml deionized water. The mixture was then finally frozen at -20°C and lyophilized to obtain the biofilm EPS in dried powder form for further analysis.

(II) Estimation of total protein content

The method used for estimating the total protein content in the biofilm of the selected strain was the Folin-Lowry method (Lowry *et al.*,1951). Bovine serum albumin (BSA) was used as standard in different concentrations (0.1- 0.5 $mgml^{-1}$) and biopolymer stock was 1 $mgml^{-1}$. 50 ml reagent A and 50 ml of (reagent B) were mixed to make the complex-forming reagent. 1 ml of freshly mixed complex-forming reagent was added to 0.2 ml of the sample or standard. The solution was left undisturbed for 10 min at room temperature. The absorbance was taken at 750 nm. The amount of total protein present in the sample was calculated from the standard curve prepared by using pure BSA as standard (Appendix II)

(III) Estimation of total sugar content

Phenol sulphuric acid method was used for total carbohydrate content estimation as described by Dubious *et al.*, (1986). To all the samples and standard 200 µl of phenol reagent (5% v/v) was added. Following addition of phenol reagent 1.0 ml of concentrated sulphuric acid was rapidly added to the surface of the solution without touching the sides of the test tube. The tubes were left undisturbed for 10 min at room temperature. After incubation the tubes were shaken vigorously and absorbance was taken after 30 min at 490 nm. The total sugar content present in the sample was calculated from standard curve (Glucose, 0- 0.5 $mgmL^{-1}$, Appendix II).

4.8 Biofilm inhibition using depolymerase enzymes

The major components of EPS as indicated by various studies is carbohydrates , while some indicate proteins to be the main constituent (Zhang *et al.*, 2001 ; Liu *et al*, 2003). Lipids also constitute a small fraction in EPS (Mattila *et al.*, 2002). EPS creates a protective matrix around the cells (Craig, 2002). Therefore the components of EPS were targeted using proteases, lipases and α-amylases for biofilm inhibition. The microtiter assay performed was according to Pitt's *et al*. (2003) with minor modifications. A 1% bacterial suspension (200µl) were added to the wells and the plates were incubated at 37°C under static conditions. Biofilm formation was observed visually. Following incubation of every 12 hrs, the supernatant was discarded, the plates were washed with sterile distilled water five times. To each well 1U/ml and 3U/ml of amylases, proteases and lipases were added. Wells without enzyme was treated as control. The plates were incubated for 1h for the reaction to take place. Plates were emptied and rewashed with sterile distilled water, 200µl of 95% ethanol was then added to fix the remaining cells for 15 min , and allowed to dry. The process was followed by crystal violet staining assay as described previously under biofilm formation assay using a modified (Merritt *et al.*, 2011). A measure of efficacy of enzymes i.e. percentage reduction was calculated.

Percentage reduction = [{(C - B) - (T- B)}/ (C-B)] * 100

Where,

C : average absorbance per well for control wells

B : average absorbance per well for blank wells

T : average absorbance per well for treated wells

4.9 Quantitative determination of viable cells

Ten fold series dilutions were made by inoculating 100µl of the bacterial suspension to 900 µl of saline solution and mixed. The aliquots (0.1 ml) were spread onto sterile Luria Agar plates and incubated for 24-48 hours at 37°C (2 plates for each dilution), viable cells were enumerated and expressed as colony forming units (CFU/ml).

Biofilm formation is a public health and cross- contamination concern, micro-organisms attach to surfaces and develop biofilm which are a concern in food and other industrial processing units. CIP and chemical disinfectants are currently been used for biofilm removal. But the two have limitations. Using CIP method removal of biofilms from joints, gaskets is not possible, on the other hand chemical disinfectants increases the probability of prduct contamination and also leads to the formation of persistors. A major role in biofilm formation is played by substratum. This study assessed the biofilm formation of the selected strains on glass, steel and PVC surfaces. These matrices were the preferred choice as they are the most commonly used in industries. The research mainly converged on the possible use of depolymerase enzymes for biofilm removal. Enzymes are preferred as they are known not to produce any harmful by products and show no corrosive action. Moreover the action of enzymes as compared to other methods is rather more specific, thus depolymerase enzyme stands as a exceptional choice for biofilm inhibition.

5.1 Isolation and screening of biofilm producing bacteria

Isolation of biofilm producing bacteria was carried out from various industrial units. A total of 200 bacterial isolates were isolated in the present study from waste water sludge samples, and various industrial sources as mentioned in the Table 5.1. Amongst the 200 isolates, 22 bacterial isolates were found to be biofilm producers. This preliminary screening was based on the morphological characteristics of the isolates, an initial basis being the mucoid appearance of the isolates. Further final screening was based on a quantitative, indirect estimation of biofilm formation via microtiter plate assay. Crystal violet stain used at 1% concentration was effective in binding with the biofilm, which was in corroboration with the findings of Djordjevic *et al.*, (2002), Wilcox (1994). Out of the 22 isolates, 4 were strong biofilm producers (Figure 5.2). Therefore the 4 strains exhibiting maximum absorbance were

preferred for further analysis. The isolates thus obtained from Federal Mogul Goetze (India) ltd, Patiala and Domestic sewage were thus named BF and BD respectively.

Table 5.1 Industrial sources, site of sample collection and isolates.

Industrial sites	**Source**	**Isolates**
Federal Mogul Goetze (India) ltd, Patiala	Fresh soil sludge	BF1
		BF2
		BF3
	Fresh soil sludge (II)	BF4
		BF5
	Grease contaminated soil	BE6
		BF7
		BF8
	Sludge STP	BF9
	Dry soil	BF10
		BF11
		BF12
		BF13
	Wet soil	BF14
	Chrome contaminated sludge	BF15
		BF16
		BF17
		BF18
Domestic sewage	Domestic sewage soil	BD1
		BD2
	Domestic sewage sludge	**BD3**
		BD4

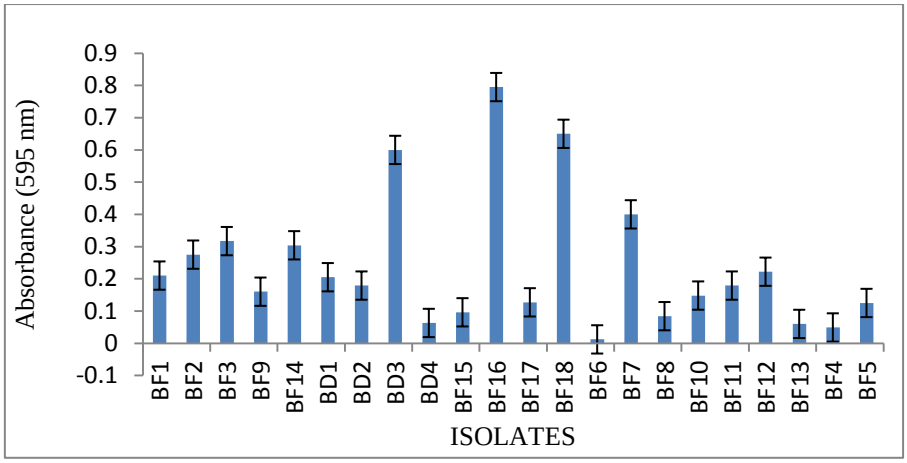


Figure 5.1 Biofilm production of 22 isolates. Each value represents the mean of 3 wells from one representative experiment. Error bars are shown as one-standard deviation.

5.2 Identification of isolates

The Table 5.2 summarizes the biochemical and morphological characteristics of the selected isolates. The four isolates i.e. BF15, BF18, BD3 and BF7 showed a positive MR test indicting that they are capable of producing stable acids. From the above results it can be stated that except BD3 all were found to be starch hydrolysing and catalase positive indicating that they can degrade hydrogen peroxide by the enzyme catalase. All the four isolates were unable to utilize sulphur containing amino acids or inorganic sulphur compounds hence they were H_2S test negative. On the contrary they were able to degrade the amino acid Tryptophan indicated by a positive indole production test. Also all of them show the absence of urease enzyme. All the four strains are gram negative and except BF7 all are motile. From the morphological and biochemical characterization, the four isolates are from Enterobacteriacae family. The selected strains were mucoid and slimy in appearance. Results of molecular characterization are awaited.

Table 5.2 Biochemical and morphological characteristics of the biofilm forming isolates.

ISOLATES / TEST	BF15	BF18	BD3	BF7
BIOCHEMICAL IDENTIFICATION				
Methyl Red Test	+	+	+	+
Voges-Proskeaur Test	-	-	-	-
Hydrogen Sulfide Test	-	-	-	-
Starch Hydrolysis	+	+	-	+
Citrate Utilization test	-	-	-	-
Indole Production Test	+	+	+	+
Urease Test	-	-	-	-
Catalase test	+	+	-	+
MORPHOLOGICAL IDENTIFICATION				
Gram Staining	-	-	-	-
Cell Morphology	rods	rods	rods	cocci
Motility	+	+	+	-
Colony Morphology	Circular, entire margin, mucoid, translucent	Large, irregular, undulate margin, mucoid	Beige, smooth, entire margin, opaque,mucoid	Circular, entire margin, mucoid, elevated

5.3 Development of biofilm kinetics

Appropriate cultivation techniques along with media composition marks the basis for all the biofilm related work. Media composition is known to play a vital role in the ability of bacteria to form biofilm (Hood *et al.*, 1997; Stepanovic *et al.*, 2004). Although it has been suggested that higher biofilm formation takes place on using minimal media (Prakash *et al.*, 2003; Gerstel *et al.*, 2001) but researchers on the contrary have reported that rich medium can also induce biofilm formation (Hood *et al.*, 1997; Stepanovic *et al*., 2004). Therefore in this study a rich medium was used. The following step is to analyze the growth and biofilm kinetics of the selected micro organism. Biofilm kinetics was done to infer the influence of

bacterial growth phase on the adhesion of cells to the surface any relation. To better understand the biofilm kinetics of the selected strains the experiments were conducted in batch cultures.the experiments were conducted in a controlled 96 well microtiter plate. Biofilm kinetics and growth kinetics were evaluated using a non-invasive, in situ method based on optical density. The biofilm kinetics of the isolates BF15, BF18, BD3, BF7 are described in Figure 5.2, 5.3, 5.4 and 5.5 respectively. The isolate BF15 exhibits maximum biofilm production with a specific growth rate (μ) of 0.12 h^{-1} in the mid and late stationary growth phases. Biofilm production occurred during the early stationary growth of the strain. Additional evidence of stationary phase cells being more adhesive than those in exponential phase is found in literature (Sharon *et al.*, 2005). The Figure 5.3 depicts that in the isolate BF18 biofilm productions initiated in the early stationary phase and exhibited maximum biofilm production in the late stationary growth phase. It exhibits a specific growth rate (μ) of 0.069 h^{-1}. The isolate BD3 exhibited biofilm production in the early stationary growth phase with a growth rate of 0.076 h^{-1} and reached maximum biofilm production level in the early senescence phase of the strain. Also its known that with time EPS production increases thereby facilitating biofilm formation over a time span (Frank *et al.*, 2003; Olofsson *et al.*, 2003; Tsuneda *et al.*, 2003). The isolate BF7 exhibited biofilm production in the late exponential and early stationary growth phases. Biofilm production reached a plateau in the late stationary growth phase. It not only showed a lower biofilm formation but also a lower specific growth rate i.e. 0.04 h^{-1} in comparison to the other three isolates. Several studies have reported dependence of biofilm kinetics on the late exponential and early stationary growth phase (Jones *et al.*, 2003; Ong *et al.*, 1999; Walker *et al.*, 2007; Sharon *et al.*, 2005)

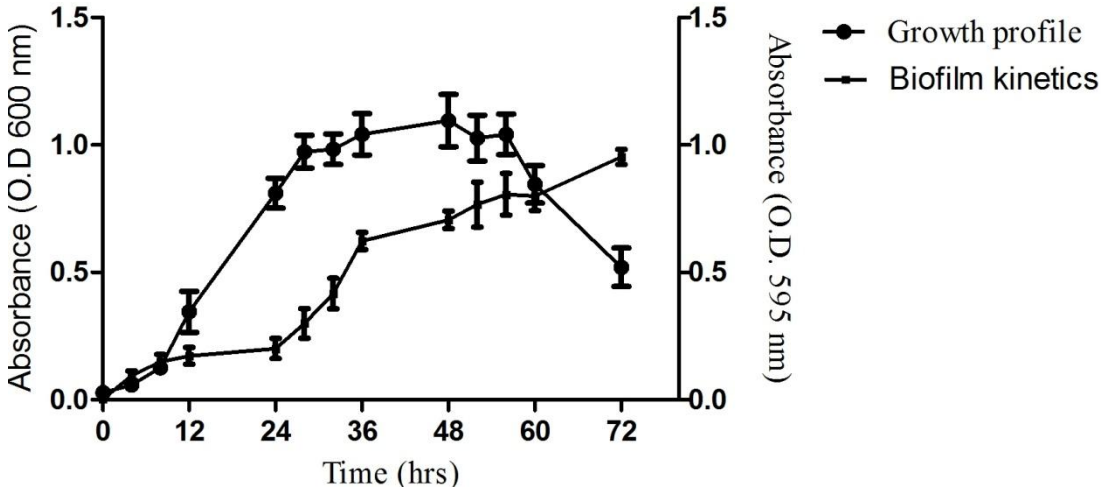


Figure 5.2 Biofilm formation and growth profile of the isolate BF15 with time. Error bars indicate the standard deviation from mean value of triplicate experiments.

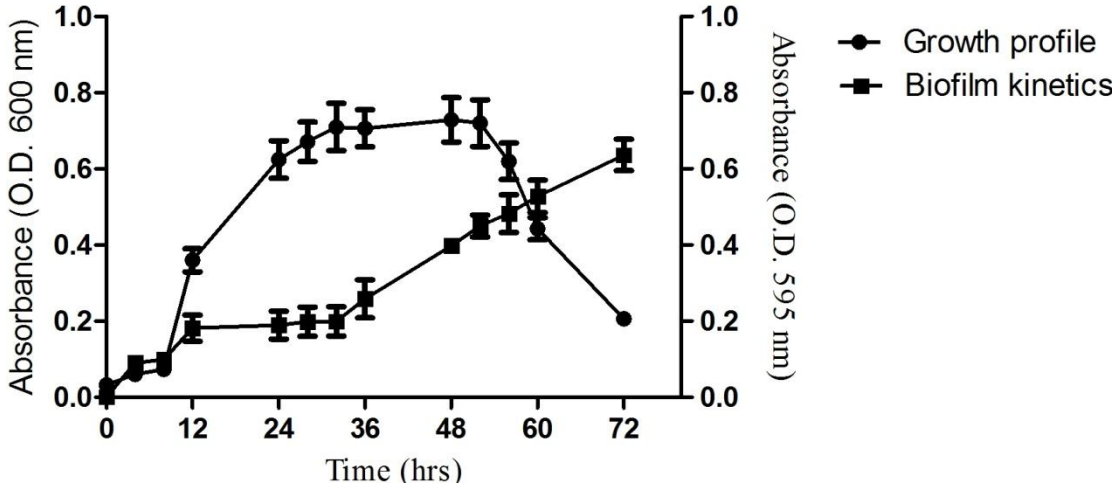


Figure 5.3 Biofilm formtion & growth profile of the isolate BF18. Error bars indicate the standard deviation from mean value of triplicate experiments.

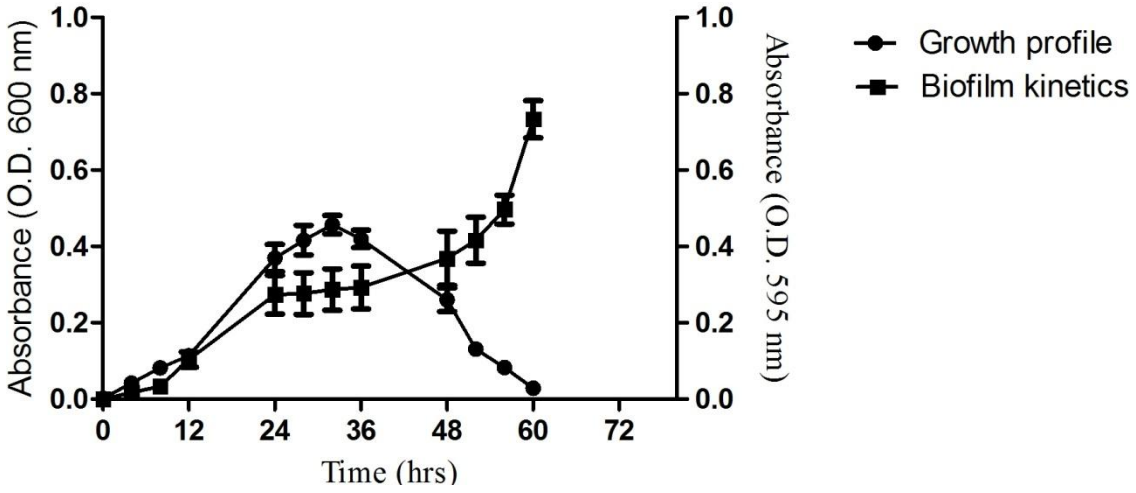


Figure 5.4 Biofilm formation & growth profile of the isolate BD3. Error bars indicate the standard deviation from mean value of triplicate experiments.

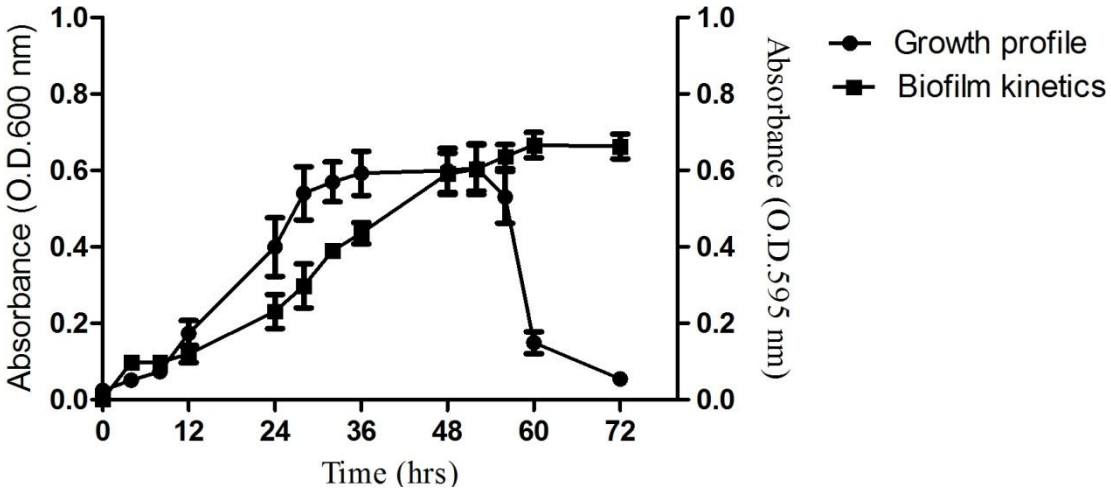


Figure 5.5 Biofilm formation & growth profile of the isolate BF7. Error bars indicate the standard deviation from mean value of triplicate experiments.

5.4 Simulation studies on various matrices

Simulation studies were carried out to observe the effect of substratum on the biofilm formation. The attachment and survival of bacterium within a biofilm is somehow linked with the interactions of the cell with the surface (Mohmmad *et al.*, 2010). The three surface materials used in the study were PVC, glass and steel surfaces. Microbial growth and biofilm

formation is known to cause detrimental effects in all the above mentioned processing premises. The efficiency of attachment of the strains on the matrices was qualitatively observed using the crystal violet staining assay, as crystal violet staining is rapid method for qualitative analysis of biofilm formation without its destabilization. The biofilm formation was observed on the above mentioned surface over a time span of 72 hours. The biofilm formation of the strains BF15, BF18, BD3 and BF7 on glass, steel and PVC surface were shown in Figure 5.6 (a-c), 5.7 (a-c), 5.8 (a-c) and 5.9 (a-c). These four strains i.e. BF15, BF18, BD3 and BF7 showed a high degree of biofilm formation on the matrices used. The biofilm so formed not only showed a gradient with respect to time but also consistency. Biofilm formation increased with respect to time. It is observed that a higher degree of attachment is seen on PVC surface which is followed by steel surfaces which in turn is followed by the glass surface. The difference in the degree of attachment lies in two properties of the surface i.e. hydrophobicity and roughness. The extent of microbial colonization appears to increase as the surface roughness increases (Donlan *et al.*, 2002). This is because surface area is higher in rough surfaces (Characklis *et al.*, 1990; Viktoria *et a*l., 2011; Rogers *et al.*, 1994; Meyer *et al.*, 2001). Microorganisms attach more rapidly to hydrophobic, non polar surfaces like PVC than to hydrophilic materials like steel or glass (Pringle *et al.*, 1983).

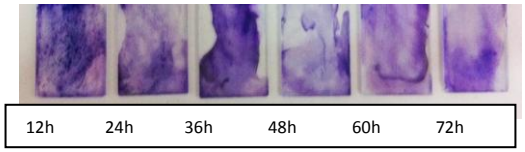


Figure 5.6 (a) glass surface

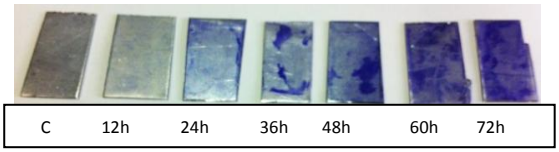


Figure 5.6 (b) Steel suface

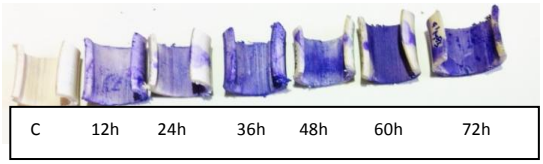


Figure 5.6 (c) PVC surface

Figure 5.6 Showing a consistent biofilm formation on (a) glass, (b) steel and (c)PVC surface by BF15 (starting from left i.e. control , 12 h, 24h…72h by the isolate).

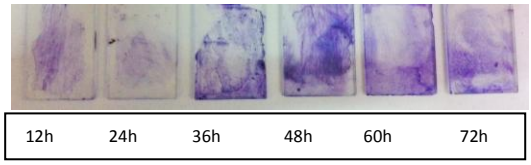


Figure 5.7 (a) glass surface

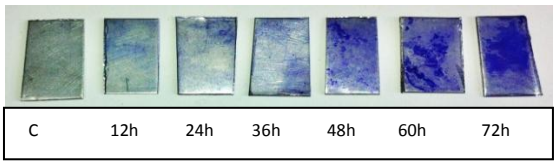


Figure 5.7 (b) Steel suface

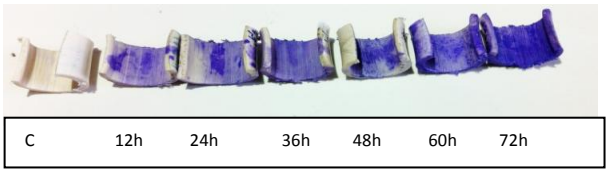


Figure 5.7 (c) PVC surface

Figure 5.7 Showing a consistency & gradient in biofilm formation on (a) glass, (b) steel and (c) PVC surface by BF18 (starting from left i.e. control , 12 h, 24h…72h by the isolate).

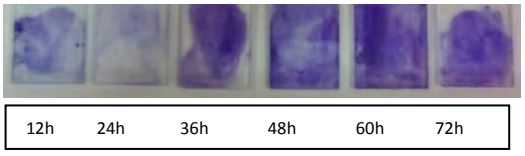


Figure 5.8 (a) glass surface

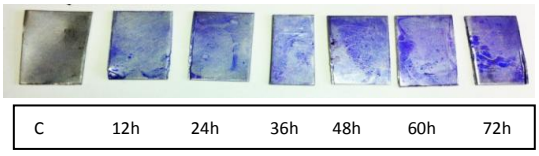


Figure 5.8 (b) Steel surface

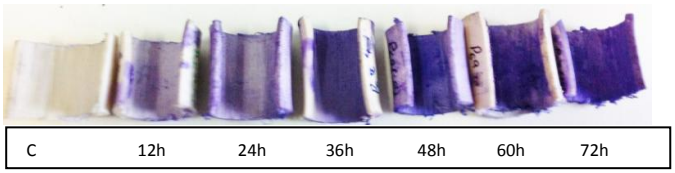


Figure 5.8 (c) PVC surface

Figure 5.8 Showing a consistent biofilm formation on (a) glass, (b) steel and (c) PVC surface by BD3 (starting from left i.e. control , 12 h, 24h…72h).

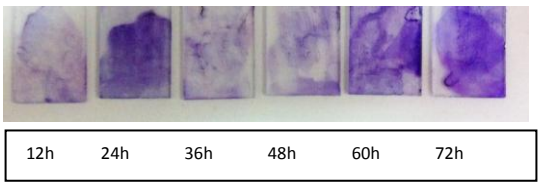


Figure 5.9 (a) glass surface

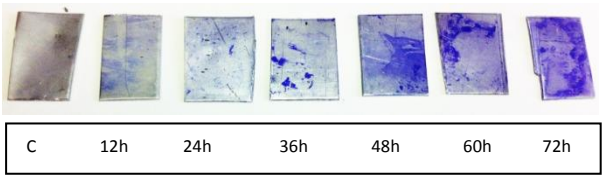


Figure 5.9 (b) Steel surface

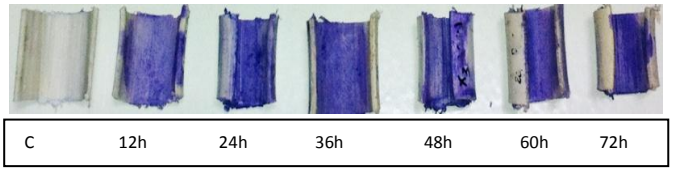


Figure 5.9 (c) PVC surface

Figure 5.9 Showing a consistent biofilm formation on (a) glass, (b) steel and (c) PVC surface by BF7 (starting from left i.e. control , 12 h, 24h…72h by the isolate).

5.5 Characterization of biofilm of the isolates

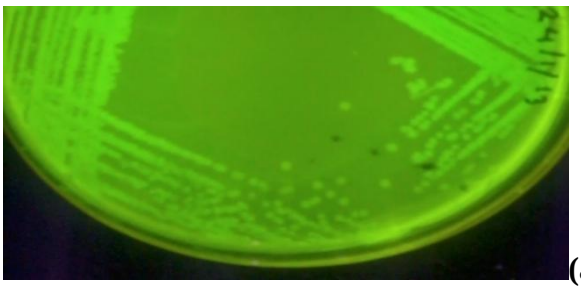
5.5.1 Nature of biofilm

In biofilm formation the cells attach themselves irreversibly to the substratum with an excess production of EPS. Almost 75% of the biofilm is EPS, comprising of polysaccharides, proteins, humic acids, lipids in general (Allison *et al.*, 2003; Tsuneda *et al.*, 2003). Exact

profile of biofilm differs with its strain. Therefore a proximate characterization of biofilm was carried out. The results of the alcian blue, sudan black and calcofluor tests are tabulated in the Table 5.3. All the four strains indicated the presence of lipids. This can be attributed to their gram negative nature, as in gram negative bacteria LPS layer is present. The isolate BD3 attested the presence of acidic polysaccharides, lipids and β linkages, credited to the positive results shown in all the three tests. The isolate BF15 exhibited the presence of the β linkage and lipids but absence of acidic polysaccharide. On the contrary both the isolate BF18 and BF7 indicated a negative result for calcofluor test. But the strain BF7 showed a presence of acidic polysaccharides, whereas BD3 exhibited a negative result for alcian blue test, indicating absence of acidic polysaccharide.

Table 5.3 Dye based biofilm characterization of the isolate

STRAIN	CALCOFLUOR	SUDAN BLACK	ALCIAN BLUE
BF15	+	+	-
BF18	-	+	-
BD3	+	+	+
BF7	-	+	+



(a)

(b)

Figure 5.10 Calcofluor assay. BF15 & BD3 showing fluorescence under UV Trans illuminator, indication a positive calcofluor test in (a) and (b) respectively.

5.5.2 Compositional analysis

It is well documented that the EPS component of biofilm varies in structure, composition among bacterial species and is dependent on the bacterial cells within the biofilm. Therefore, prior to assessing the ability of depolymerases to degrade the biofilm of the strains a quantitative analysis of biofilm of the strains was deemed necessary. Different biofilm produce not only different but a variable amount of EPS (O'Toole *et al.*, 2000). Also according to Consterton and Donlan (2002), biofilm EPS in known to perform various functions, most importantly it behaves as an secure anchor which secures the biofilm from adverse affects. It also enhances the adhesion of cells to the substrate. Therefore to select from a range of depolymerase enzymes it would be easier to select if the compositional analysis is conducted. The carbohydrate concentration of all the strains was higher than the protein content, as shown in Table 5.4. These results correspond to the studies of Steinberg *et al.,* (2004) who found more concentration of polysaccharide than protein.

Table 5.4 Proximate quantification of biofilm with respect to its constituents i.e. carbohydrate and protein fraction.

STRAIN	PROTEIN (P) (ug/ml)	SUGAR (S) (ug/ml)	P:S ratio
BF15	9	55	1:6
BF18	44	56	1:1.5
BD3	36	42	1:1
BF7	7	27	1:4

5.6 Biofilm inhibition by depolymerase enzymes

EPS is the main barrier protecting the bacterial cells within the biofilm against the inhibiting agents. Biofilms are eliminated directly as its structural component i.e. EPS is targeted by the enzymes (Xavier *et al.*, 2005). EPS is a significant target for eradicating biofilms, since it plays an important role in the structural integrity and attachment properties of biofilms (Stoodley *et al,* 2002). The structural components of EPS i.e. proteins, polysaccharides, lipids are the ultimate target of the respective enzymes. Consequently a combination of enzymes is generally thought to be useful in removing biofilm.

5.6.1 Protein depolymerisation

Proteases cleave the amide bonds effectively depolymerising the protein into monomers. Thus destroying the physical structure of the biofilm (Leslie, 2011). The proteases are also known to inhibit biofilm formation by removing the surface proteins used for adhesion by the bacterial cell (Longhi *et al.*, 2008). The proteases were used in two different concentration, 1U/ml and 3U/ml. A higher protein depolymerisation is observed in the strain BF18 showing a maximum of 69.1% reduction when 3U proteases are used which is followed by BD3 with a reduction 65.06%, BF15 following BD3 with a reduction of 57.5 %, which is followed by BF7 showing a percent reduction of 45.6%. This result is in accordance with the concentration of protein present in the biofilm of the selected strains i.e. higher the protein concentration higher is the percent reduction using proteases.

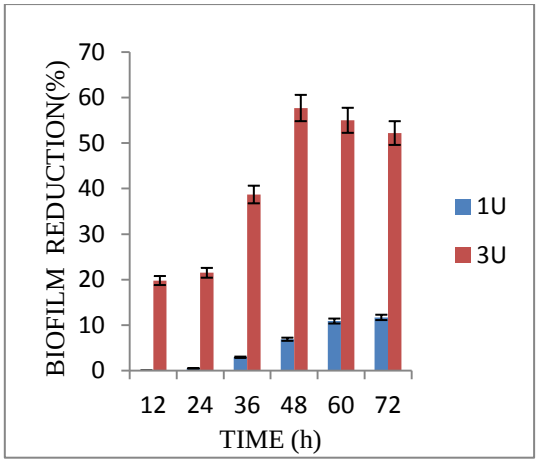


Figure 5.11 (a)

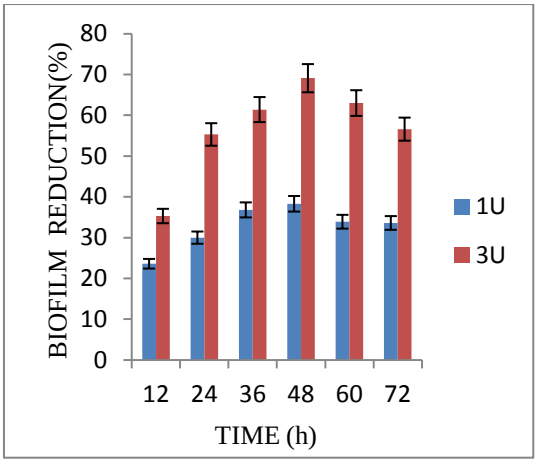


Figure 5.11 (b)

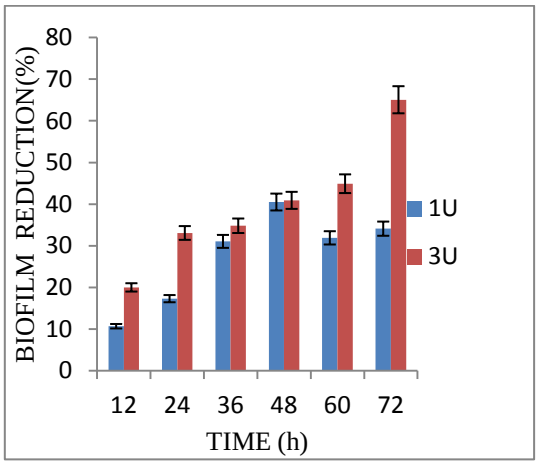


Figure 5.11 (c)

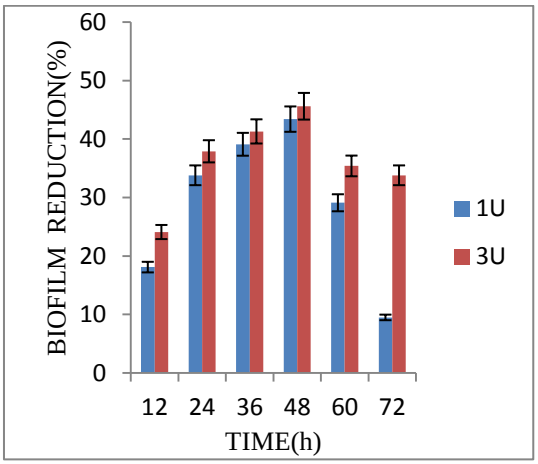


Figure 5.11 (d)

Figure5.11 (a-d) shows the percentage reduction of the biofilm of the strain BF15, BF18, BD3 and BF7 using proteases of 1U and 3U concentration at different time intervals. Biofilm reduction is expressed in percentage with error bars indicating standard deviation.

5.6.2 Polysaccharide depolymerisation

Structural heterogeneity of the EPS dictates the neccessity to explore the efficiency of a range of depolymerase enzymes. A compositional analysis of the structure of EPS of the biofilm indicatrs the use of amylases due to the presence of sugars in higher concentration as compared to proteins. Amylases were chosen as they have the ability to cleave 1,4-glycosidic

bond. Further as α-amylases are are more effective in comparison to β- and γ-amylases (Craigen B. *et al.*, 2011), α-amylases were used to assess its efficiency in biofilm inhibition. In BF18 66.6% polysaccharide depolymerisation was observed on using α-amylases of 3U concentration. Reduction of 52% was observed in BF15, which was then followed by the strain BD3 and BF7 with 49.9 % and 45% reduction respectively. Also as mentioned before the strain BD18 also shows the presence of a higher sugar concentration, therefore showing maximum percent reduction with α- amylases.

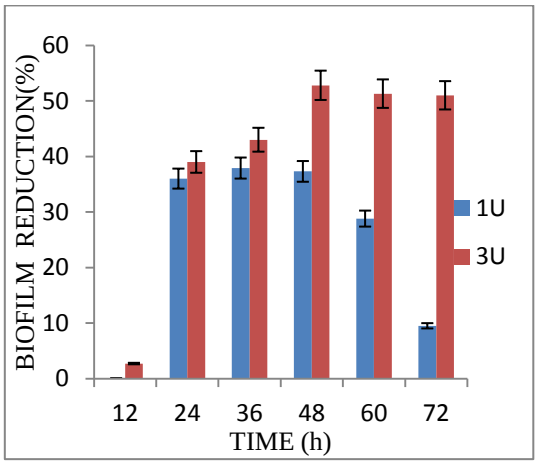


Figure 5.12 (a)

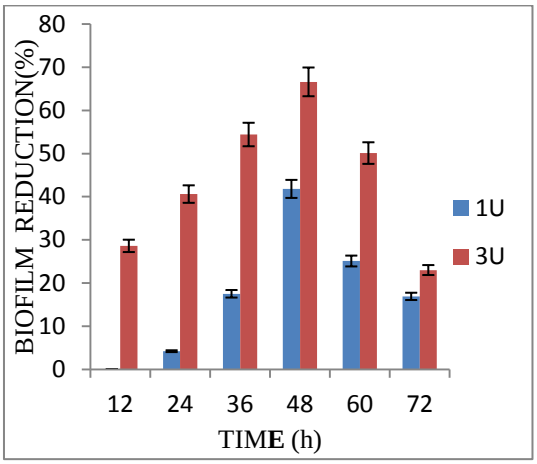


Figure 5.12 (b)

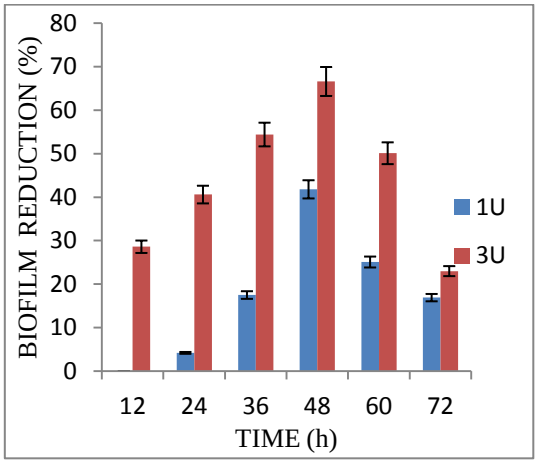


Figure 5.12 (c)

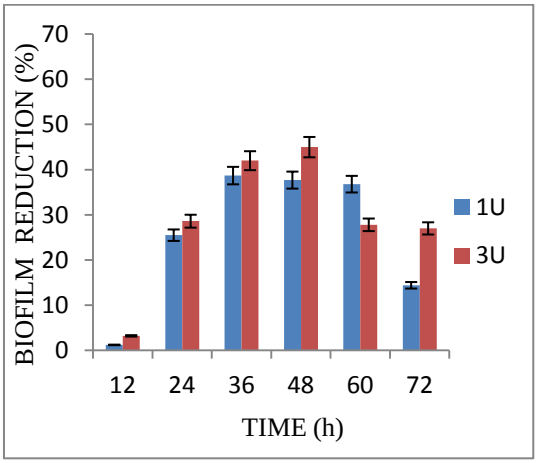


Figure 5.12 (d)

Figure 5.12 (a-d) shows the percentage reduction of the biofilm of the strain BF15, BF18, BD3 and BF7 respectively using a 1U and 3U concentration of α-amylases at different time

intervals. Biofilm reduction is expressed in percentage with error bars indicating standard deviation.

5.6.3 Lipid depolymerisation

Lipids, as one of the structural components of EPS plays an important role in gram negative bacterial biofilm formation (Tielen *et al.*, 2010; Mattila *et al.*, 2002, O' Toole *et al.*, 2000, O'Toole et al., 2003). Therefore lipids were targeted as they are one of the components of EPS of the biofilm. Higher lipid depolymerisation was observed in BD3 with a percent reduction of 73.6% which was followed by a 62.9% reduction by the strain BF18, and finally by the strain BF7 and BF15 with 52.02% and 39.9% reduction respectively.

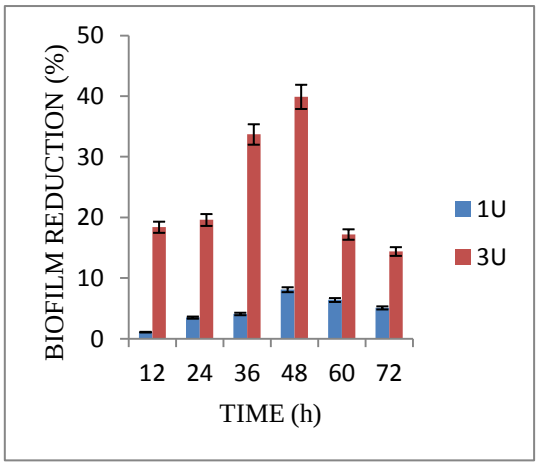
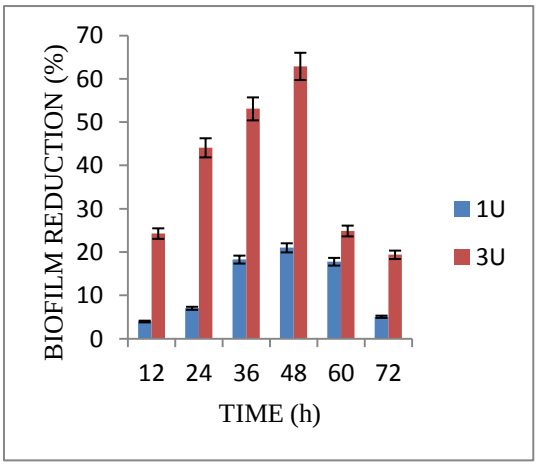


Figure 5.13 (a)



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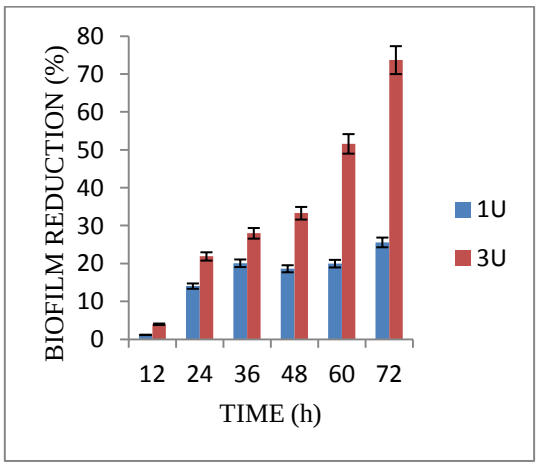

Figure 5.13 (c)

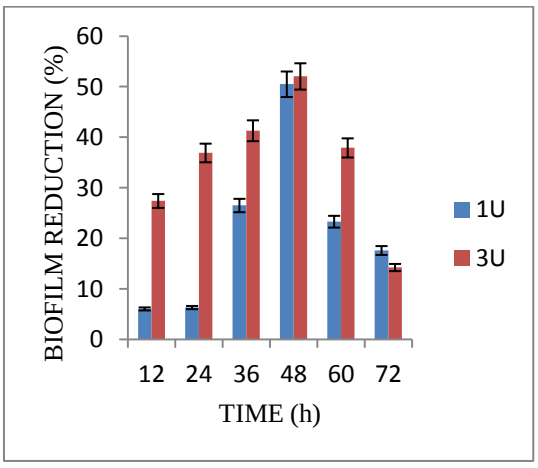

Figure 5.13 (d)

Figure 5.13 (a-d) shows the percentage reduction of the strain BF15, BF18, BD3 and BF7 respectively using 1U and 3U concentration of lipases at different time intervals. Biofilm reduction is expressed in percentage with error bars indicating standard deviation.

5.6.4 Cumulative effect of depolymerases

With proteases, α-amylases and lipases varying effect on the biofilm inhibition were observed. The target area of all these as mentioned before is the structural components of the biofilm EPS. The enzymes when used in conjunction are known to eliminate the existing biofilms and prevent any further formation (Leslie *et al.*, 2011). Therefore the effect of cumulative depolymerases were studied on the biofilms formed by the strains. A cocktail of enzyme was prepared both of 1U and 3U concentration and its effect was seen on each of the strain. The effect of cocktail enzyme was higher than the individual enzymes used in 3U concentration and least effect was seen when 1U enzyme concentration was used. A maximum reduction of 80% was observed in the isolate BD3, which is followed by the isolate BF18 showing a percent reduction of 71.6%, BF7 and BF15 follows the isolate BF18 with a reduction of 62.01% and 59.9% respectively.

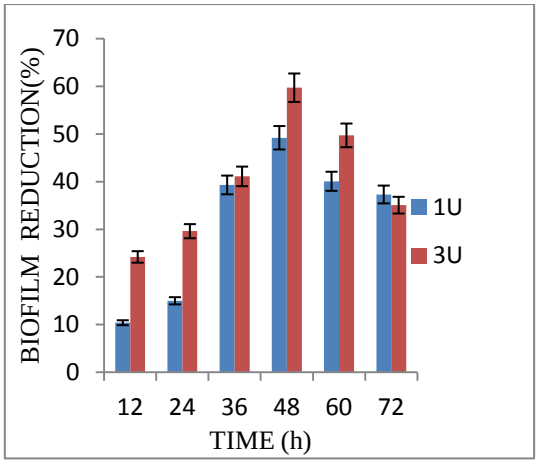

Figure 5.14 (a)

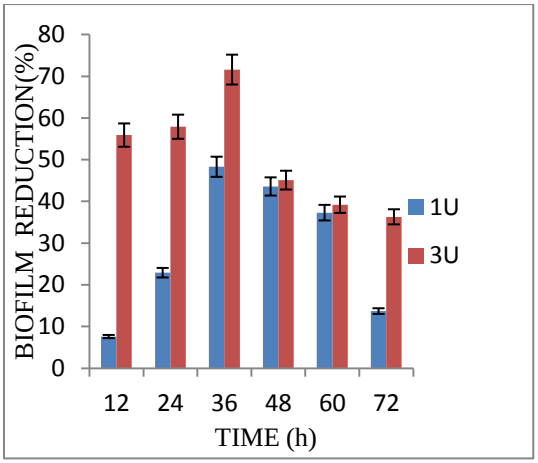

Figure 5.14 (b)

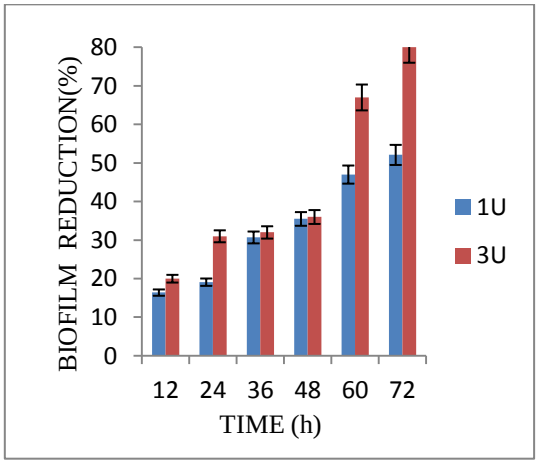

Figure 5.14 (c)

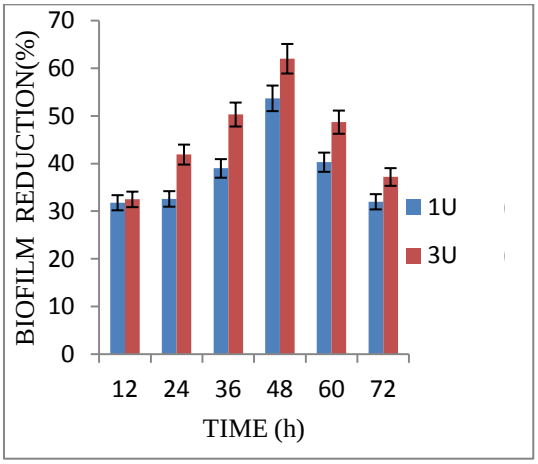

Figure 5.14 (d)

Figure 5.14 (a-d) shows the percentage reduction of the strain BF15, BF18, BD3 and BD7 using a cocktail of enzymes with 1U and 3U concentration respectively. Biofilm reduction is expressed in percentage with error bars indicating standard deviation.

5.6.5 Quantitative determination of viable cells

The viability of the isolates decrease after the enzymatic treatment. Maximum decline in the viability was shown by the isolate BD3 which was followed by BF18, BF15 and BD7 respectively (Table 5.5)

Table 5.5 Comparison of viable cells of treated (with enzyme) and untreated biofilm of the isolates

	BF15	BF18	BD3	BD7
Unreated (CFU/mL)*	1.90±1.6	2.40±1.8	1.5±1.7	2.02±1.6
Treated (CFU/mL)*	0.8±1.2	1.2±1.9	0.4±1.1	1.01±1.3

*viable cells(CFU/mL) × 10^5 Average± Standard Deviation

6. CONCLUSION

The overall objective of this work was to study the effect of depolymerase enzymes on biofilm inhibition. Four biofilm producing bacterial isolates BF15, BF18, BF7 and BD3 (Enterobacteriace family) were screened from different sludge and soil samples and biofilm formation was analysed. Initiation of biofilm formation in liquid medium was observed at 24 hours whereas in case of solid medium (steel, glass surface and PVC) time of initiation was from 24 to 36 hours. Maximum biofilm formation was observed on PVC in 24 hours, which can be attributed to the increase in the roughness and hydrophobicity of the surface.

A comprehensive characterization of biofilm properties of the isolates was carried out. The compositional analysis of biofilm revealed carbohydrate as major constituent followed by proteins and minimal amount of lipids. The strain BF15 exhibited maximum biofilm formation, whereas the strain BF7 produced the lowest biofilm as indicated by the crystal violet assay. The biofilm of the isolate BF15 was composed of higher polysaccharides in comparison to that of proteins in the ratio 6:1 .

On the basis of compositional analysis of biofilm, depolymerase enzymes (Proteases, α-amylases, lipases) were selected for biofilm removal studies. Lipase inflicted maximum biofilm reduction of 74%, amylases and proteases exhibited almost similar biofilm reduction of 66.6% and 69.1% respectively. The results of inhibition studies suggested lipases to be more effective in biofilm reduction. On the other hand if these are used in combination, a notable reduction of 80% was observed, indicating that the cocktail effect of these depolymerase enzymes seems to be promising for biofilm reduction.

Based on the above studies, it can be inferred that an approach using these enzymes may be a possible environment friendly solution for biofilm removal. However, for feasibility of these findings, further studies evaluating the effects of external factors, pH, temperature, flow velocity, total dissolved solids are mandatory.

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APPENDIX I

Luria Bertani Agar (pH 7.5 $\pm$ 0.2)

Composition	gL^{-1}
Agar	15.0
Tryptone	10.0
NaCl	10.0
Yeast extract	5.0

Luria Bertani Broth (pH 7.5 $\pm$ 0.2)

NaCl	10.0
Pancreatic digest of Casein	10.0
Yeast extract	5.0

SIM Agar (pH 7.3 ± 0.2)

Peptone	30.0
Beef extract	3.0
Ferrous ammonium sulphate	0.2
Sodium thiosulfate	0.025
Agar	3.0

Starch Agar (pH 7.2 ± 0.2)

Peptone	5.0
Beef extract	3.0

Starch	2.0
Agar	15.0

Simmons Citrate Agar (pH 6.6 ± 0.2)

Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Magnesium sulfate	0.2
Agar	15.0
Bromothymol blue	0.08

Trypticase soy agar (pH 7.3 ± 0.2)

Trypticase	15.0
Phytane	5.0
Sodium chloride	5.0
Agar	15.0

REAGENTS

Folin-Lowry reagent

Reagent A (Alkaline solution)

Na_2CO_3	2.0
NaOH	0.2

Reagent B

$CuSO_4$	0.25
Na-K-tartarate	0

Barritt's Reagent

Solution A

5.0 gm of α- napthol dissolved in 95.o ml of absolute ethanol.

Solution B

- 40.0 gm of KOH dissolved in 100 ml of distilled water.

Kovac's reagent

10 g of p-aminobenzaldehyde dissolved in 150 ml of isoamylalcohol and then slowly adding 50 ml of concentrated hydrochloric acid.

CHEMICALS

Methyl red solution

0.1 gm methyl red dissolved in 95% absolute ethanol. Diluted to 500 ml with distilled water

1% Crystal violet

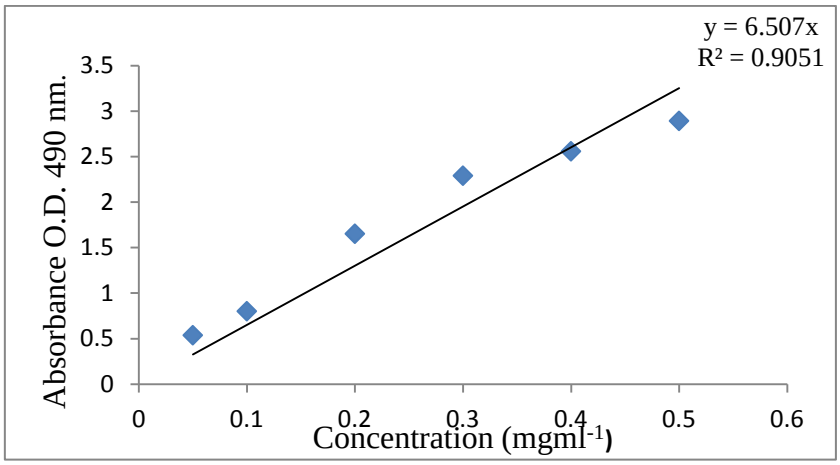
0.1 gm crystal violet in 100 ml of distilled water.

30 % Acetic acid

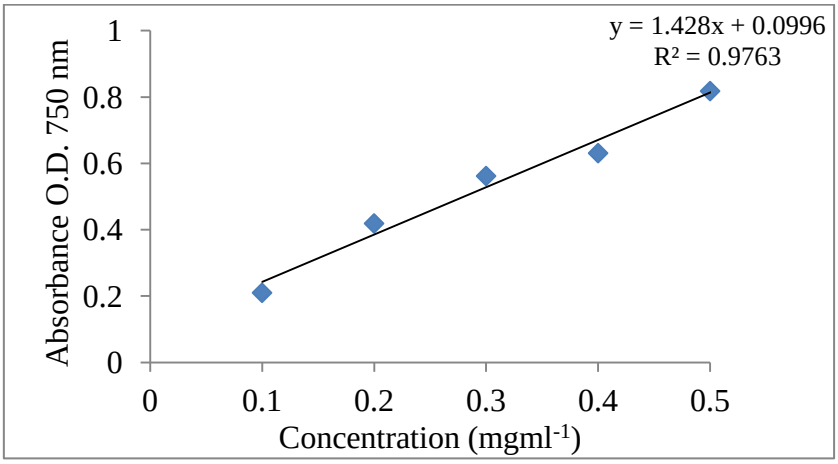
30 ml Acetic acid dissolved in 70 ml distilled water.

0.02 % Sudan Black B

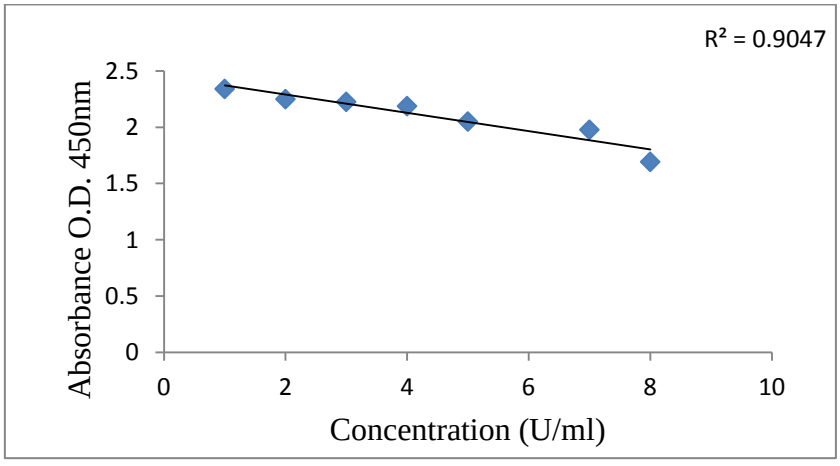
0.02 gm of Sudan Black dissolved in 100 ml of distilled water



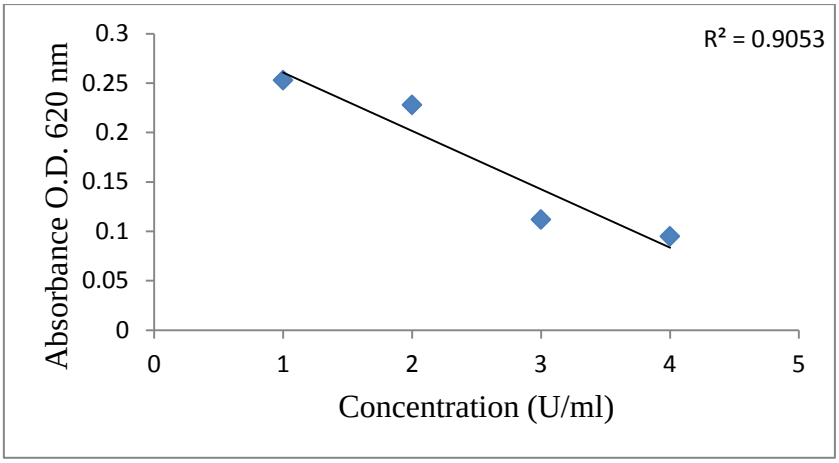
Standard curve of Glucose (0-0.5 mgmL^{-1})



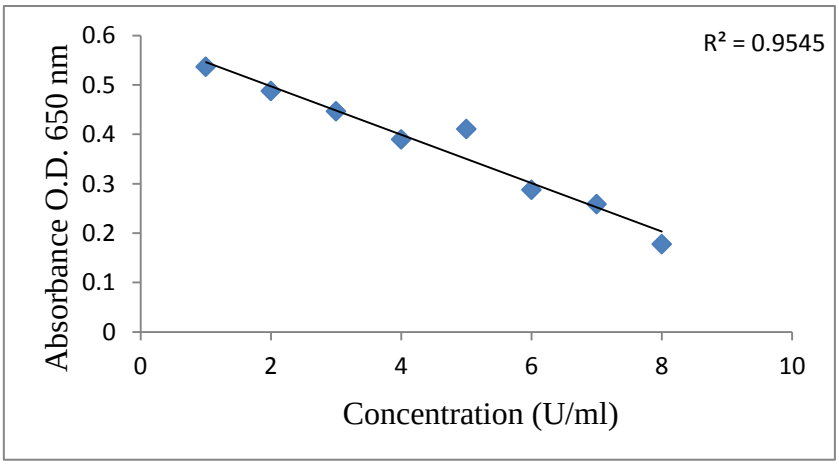
Calibration curve of protein using BSA.



Standard curve of enzyme Lipase with using tributyrin as substrate.



Standard curve of the enzyme α-amylase using starch as the substrate.



Standard curve of the enzyme protease using casein as the substrate.