STUDIES OF MICROBIAL BIOFILM DEVELOPMENT AND THEIR ERADICATION USING VARIOUS ENZYMES

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CERTIFICATE

This is to certify that the work titled **"STUDIES OF MICROBIAL BIOFILM DEVELOPMENT AND THEIR ERADICATION USING VARIOUS ENZYMES"** submitted by "SRISHTI MEHROTRA" in partial fulfillment for the award of degree of B. Tech., of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Dr. SAURABH BANSAL

(Assistant professor)

Date: 26-05-2014

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SUMMARY

Microbial biofilms that grow on abiotic surface can be found in various settings including natural, industrial and hospitals and forms a major area for research owing to the properties exhibited by them which are distinct from that of a planktonic cells. Owing to the heterogeneous structural composition of the biofilm, it is likely that multiple mechanisms might be in action within a single community. Microbial biofilms are of special concern to the food and fermentation industry, as biofilms on raw materials or food contact surfaces represent possible sources of product contamination with spoilage or pathogenic microorganisms. Need for research in the field of biofilm eradication has thus gained a substantial momentum in past two decades. Numerous resources have being exploited for their potential usefulness against biofilm removal and have failed in ensuring the complete removal of the biofilm.

Break down of the structural components of the EPS (Extracellular Polymeric Substance) can be the possible targets to ensure the successful eradication of biofilm. Enzymes have been used and proven useful for the degradation of the multi-structural EPS of the biofilm. The mode in which enzymes destroy the EPS is by degrading the physical integrity of the EPS hence unraveling the entire structure. The aim of our study included:

- Formation of biofilm
- Action of different enzymes on the bacterial biofilm

The motive behind doing so is to determine the kind and quantity of the enzyme that is active in degrading the biofilm while keeping the related hazard and the cost to the minimum.

INTRODUCTION

Microbial biofilms play a crucial role in a variety of disciplines, including biotechnology, immunology, biofouling and biodeterioration (Fleming et al., 2001; Dunne 2002). A biofilm is an aggregate of microorganisms in which cells are stuck to each other and/or to a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS). Biofilm EPS, which is also referred to as "slime," is a polymeric jumble of DNA, proteins and polysaccharides. They are a common mode of bacterial growth in nature and their presence has an enormous impact on many aspects of our lives, such as sewage treatment, corrosion of materials, food contamination during processing, pipe collapse, plant-microorganisms interaction in the biosphere, the formation of dental plaque, the development of chronic infections in live tissue (mastitis, Otitis, pneumonia, urinary infections, osteomyelitis) or problems related to medical implants.

Literature includes some evidence that cell contact with surfaces stimulates transcription of the EPS genes (Dunne, 2002). Monitoring the EPS gene expression in adherent populations enables a better understanding on the basis of biofilm phenotype (Allison et al., 1997; Peterson et al., 2005). 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 (Dunne 2002; Pontefract, 1991). The production of EPS by attached microorganisms is a very complicated process, which is affected by many unique parameters. It is also considered that the mechanisms of biofilm development process are vastly different from species to species (Dunne, 2002). Despite the difficulties associated with the study of the production of EPS by anchored cells, analysis of all described data can enable control of the microbial adhesion process in different environments.

In medical scenario the biofilm has been found to interfere with clinical therapy for chronic, persistent and wound-related infections on various indwelling medical devices (Fux et al. 2005; Hall-Stoodley & Stoodley 2009). Biofilms also trigger inflammation and impair the wound-

healing process (Wolcott et al. 2010). Often, the only effective treatment option for biofilm-based chronic wound infections is to amputate the infected limb (Jeys & Grimer 2009). All of these factors pose significant challenges to clear infections and compel the development of new methods designed to inhibit bacterial biofilm formation. Although research on biofilms has surged over the past few decades, the majority of biofilm research to date has focused on external biofilms, or those that form on various surfaces in our natural environment.

Over the past years, as scientists developed better tools to analyze external biofilms, they quickly discovered that biofilms can cause a wide range of problems in industrial environments. For example, biofilms can develop on the interiors of pipes, which can lead to clogging and corrosion. Biofilms on floors and counters can make sanitation difficult in food preparation areas. Since biofilms have the ability to clog pipes, watersheds, storage areas, and contaminate food products, large companies with facilities that are negatively impacted by their presence have naturally taken an interest in supporting biofilm research, particularly research that specifies how biofilms can be eliminated.

This means that many recent advances in biofilm detection have resulted from collaborations between microbial ecologists, environmental engineers, and mathematicians. This research has generated new analytical tools that help scientists to identify biofilms.

COMPOSITION OF EPS

The EPS matrix is generally from 0.2 to 1.0μm thick. In some bacterial species, the thickness of the EPS layer does not exceed values from 10 to 30nm (Sleytr, 1997). The chemical structure of polymeric substances secreted by the cells into the environment is diversified. EPS compounds belong to such different classes of macromolecules as polysaccharides, proteins, nucleic acids, glycoproteins and phospholipids (Sutherland, 2001; Branda et al., 2005). Among one bacteria species, EPS compounds may also belong to different categories. These microorganism features are often used during cell identification and classification procedures. In addition the usage of the antigenic properties of the extracellular molecules enables the serological characterization of the cells.

Most microbial exogenous layers contain neutral carbohydrates (mainly-hexose, seldom-pentose) and uronic acids. The commonest extracellular carbohydrates substituents are acetate esters, pyruvtes, formates and succinates. The presence of polypeptides in the EPS matrix is the feature of a very few Gram-positive bacteria cells. The best-investigated components of the EPS layer are polysaccharides and proteins (Sleytr, 1997; Sutherland, 2001).

BIOFILM FORMATION AND STAGES INVOLVED DURING BIOFILM DEVELOPMENT

THE PRIMARY STAGE

The primary adhesion stage constitutes the beneficial contact between a conditioned surface and planktonic microorganisms. During the process of attachment, the organism must be brought into close proximity of the surface, propelled either randomly or in a directed fashion via chemotaxis and motility (Prakash et al., 2003). Once the organism reaches critical proximity to a surface, the final determination of adhesion depends on the net sum of attractive or repulsive forces generated between the two surfaces. These forces include electrostatic and hydrophobic interactions (Melo et al., 1997; Kumar et al., 2006) and van der Waals attractions (Denyer et al., 1993). This attachment is unstable and reversible and if the environment is not favorable for microbial attachment, cells can detach from the surface (Ghannoum and Otoole, 2004). The solid liquid interface between a surface and an aqueous medium provides an ideal environment for the attachment and growth of microorganisms. Attachment occurs mostly on surfaces that are rougher, more hydrophobic (Palmer et al., 1997) and coated by conditioning films.

The primary stage is reversible and is characterized by a number of physiochemical variables that define the interaction between the bacterial cell surface and the conditioned surface of interest (An et al., 2000; Singh et al., 2002; Liu et al., 2004). When a biofilm is composed of heterogeneous species, the metabolic byproducts of one organism might serve to support the growth of another while the adhesion of one species might provide ligands which allow the attachment of others (Dunne, 2002). Conversely, the depletion of nutrients and accumulation of

toxic byproducts generated by primary colonizers may limit the species diversity within a biofilm (Marsh, 1995).

THE SECONDARY STAGE

The secondary stage involves anchoring of bacteria to the surface by molecular mediated binding between specific adhesions and the surface (kumar et al., 2006). In this process loosely bound organisms gather together and produce exopolysaccharides that complex with surface materials (An et al. 2000; Rachid et al., 2000; Li et al., 2007). Once the bacteria have attached irreversibly to the surface they undergo a range of genotypic and phenotypic changes to ensure the development and maturation of the biofilm. All bacteria produce multiple adhesions some of which are regulated at the transcriptional level, depending on the genes encoded, permitting organisms to switch from sessile to planktonic forms under different environmental influences (Li et al., 2007). A good example of this phenomenon is that of *Staphylococcus epidermidis*, which produces a polysaccharide intracellular adhesion (PIA) that is essential for cell to cell adhesions and biofilm formation (Dunne, 2002).

The changes described above result in the production of increased amounts of EPS, increased resistance to antibiotics, increased UV resistance, gene exchange events that are produced (O'Toole et al., 2000). With certain organisms, several distinct adhesions might be used for surfaces attachment depending on the environment (O'Toole et al., 2000).

Various structures such as flagella, fimbraie, outer membrane proteins (OMPs), curli (a proteinaceous surface structure) and extracellular polymers structure (EPS) are involved in biofilm formation (Watnick et al., 1999). They have distinct roles in different species and under different environmental conditions (Giaouris et al., 2006). Flagella motility is important to overcome the forces that repel bacteria from reaching many abiotic materials. Once it reaches the surfaces, appendages such as pili, OMPs and curli are required to achieve stable cell-to-cell and cell-to-surface attachments. Flagella apparently play an important role in the early stages of

bacterial attachment by overcoming the repulsive forces associated with the substratum (Giaouris et al., 2006).

Korber et al. (1989) used motile and non-motile strains of *Pseudomonas fluorescens* to show that motile cells attach in greater numbers and against the flow more rapidly than do non-motile strains. Non-motile strains do not recognize the substratum as evenly as motile strains, resulting in slower biofilm formation by non-motile organism (Prakash et al., 2003). A number of aquatic bacteria possess fimbraie, which have also been shown to be involved in bacterial attachment to animal cells (Meyer, 2003; Prakash et al., 2003, Giaouris et al., 2006).

MICRO COLONY FORMATION

After the adherence of bacteria to the inert surface, the association becomes stable for micro colony formation (Palmer et al., 1997; O'toole et al., 2000). The bacteria begin to multiply while sending out chemical signals that intercommunicate among the bacterial cells. Once the signal intensity exceeds a certain threshold level, the genetic mechanisms underlying exopolysaccharide production are activated. In this way, the bacteria multiply within the embedded exopolysaccharide matrix, thus giving rise to formation of micro colony (Prakash et al., 2003).

Micro-colonies further develop into macro-colonies which are divided by fluid filled channels and enclosed in an extracellular polysaccharide matrix (Allison, 2003). Macro-colonies, compared to micro-colonies, are composed of some bacteria cells and are enclosed in an extracellular matrix and have a higher metabolic and physiological heterogeneity (Ghannoum and O'Toole, 2004). In the non-motile *Staphylococcus epidermidis*, polysaccharide and protein adhesions were linked for the attachment of this bacterial species, while a novel biofilm associated protein was found to be involved in attachment and intercellular adhesion of *S. aureus* (Rupp et al., 1991).

FORMATION OF THREE DIMENSIONAL STRUCTURES

During the attachment phase of biofilm development, the transcription of specific genes takes place. These are required for the synthesis of EPS (Prakash et al., 2003). Attachment itself can initiate synthesis of the extracellular matrix in which the sessile bacteria are embedded followed by formation of water filled channels in the circulatory system that helps in delivering nutrients to and removing waste products from the cell communities in the micro colonies (Prakash et al, 2003).

BIOFILM MATURATION

Once bacteria have irreversibly attached to a surface, the process of biofilm maturation begins. The overall density and complexity of the biofilm increases as surface bound organisms begin to actively replicate and extra cellular components generated by attached bacteria interact with organic and inorganic molecules in the immediate environment to create glycocalyx (Carpentier et al., 1993). The availability of nutrients in the immediate environment within the biofilm and the removal of waste, limits the growth potential of any bacterial biofilm (O'toole et al., 1998; Otoole et al 2000). In addition, there is an existence of an optimum hydrodynamic flow across the biofilm that determines the maximum growth (Carpentier et al., 1993). Other factors that control biofilm maturation include the internal pH, oxygen carbon source, osmolarity, temperature, electrolytic concentration and the flux of materials and surface types. The surface types can be either.

- High surface energy materials that are negatively charged hydrophilic materials such as glass, metals or minerals.
- Low surface energy materials that are either low positively or low negatively charged; hydrophobic materials such as plastic made up of organic polymer (O'Toole et al., 1998).

At some point, the biofilm reaches a critical mass and a dynamic equilibrium is reached at which the outermost layer of growth begins to generate planktonic organisms. These organisms are free to escape the biofilm and colonize other surfaces. Cells nearest the surface

become inactive or die due to a lack of nutrients, decrease in p, pO_2 or an accumulation of toxic metabolic byproducts (Dunne, 2002).

The primary development, maturation and the breakdown of a biofilm might be regulated at the level of population density dependent gene expression controlled by cell-too cell signaling molecules such as acylated homoserine lactones(Stickler et al., 1998). Once fully matured, a logical cooperation and metabolic efficiency provides a form of functional communal coordination that mimics primitive eukaryotic tissues (Costerton et al., 1995).

DETACHMENT AND DISPERSAL OF BIOFILM CELLS

As the biofilm gets older, cells detach and disperse and colonize a new niche. This detachment can be due to various factors including, fluid dynamics and shear effects of the bulk fluid (Brugnoni et al., 2007). Some bacteria are shed from colony and some stop producing EPS and are released into the surrounding environment (Herrera et al., 2007). Biofilm cells may be dispersed either by shedding of daughter cells from actively growing cells or detachment as a result of nutrient levels (Spiers et al., 2003). The released microorganisms may be transported to new locations and restart the biofilm process (Prakash et al., 2003).

As the thickness of the EPS increases, anaerobic conditions develop within the biofilm. Because of the film thickness and the activity of anaerobic species, the film detaches and sloughs off from the surface of the substrate. Polysaccharides degrading enzymes specific for EPS degradation for different organisms may be produced during different phases of biofilm growth and contribute to detachment.

In previous studies, it was mentioned that several bacterial species can synthesize polymer degrading enzymes to control the production of the EPS. *P. fluorescens* and *P. aeruginosa* were indicated to produce enzymes known as lyases which can degrade their exopolysaccharides and lead to the detachment of the cells from the surface (Boyd and Chakrabarty, 1994)

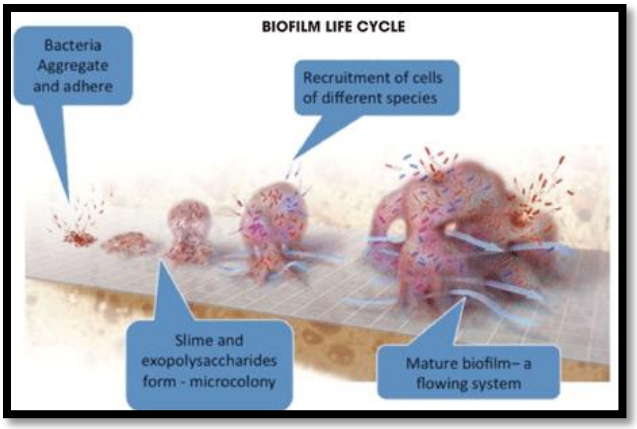


Figure 1 schematic representation of biofilm formation and development

ABOUT THE STRAINS

Many strains been studied for their biofilm developing characteristics. In this project we have chosen to work on the following biofilm forming bacterial strains.

Escherichia coli

Escherichia coli were first recognized as an enteric pathogen in 1982 (Riley et al, 1983). It has since been characterized in several laboratories as causing self-limiting diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura in children and other susceptible groups of individuals (Doyle, M.P 1991;Meng et al., 2001). Outbreaks of *E. coli* O157:H7 infections have been primarily associated with eating undercooked ground beef, but a variety of other foods have also been implicated as vehicles.

E. coli is known to produce exopolysaccharides (EPS) (Grant et al., 1969; Junkins et al., 1992; Mao et al, 2001), which can provide a physical barrier to protect cells against environmental stresses. EPS is also involved in cell adhesion and biofilm formation (Frank, J.F 2000; Wiener 1995). EPS can serve as a conditioning film on inert surfaces, affect cell attachment by functioning as an adhesive or anti-adhesive (Oflek and Doyle, 1992), and influence the formation of three-dimensional biofilm structures (Danese et al., 2000).

E. coli O157:H7 has also been shown to produce curli, a thin, coiled fimbriae-like extracellular structure.

Staphylococcus aureus

Staphylococcus aureus is widely involved in minor to severe infections. *S. aureus* has a wide range of virulence factors and can cause acute and chronic infections at many anatomical sites. In the last 2 decades, multi drug resistant *S. aureus* and methicillin-resistant *S. aureus* (MRSA) have emerged as major causes of hospital-, community-, and livestock- acquired infections, which are increasingly difficult to manage (Diep et al.,2006, Gould et al., 2012).

Two stages of staphylococcal biofilm formation have been described. The first stage involves attachment of cells to a surface. This stage of biofilm formation is likely to be mediated in part by cell wall-associated adhesins, including the microbial surface components recognizing adhesive matrix molecules. The second stage of biofilm development includes cell multiplication and formation of a mature structure consisting of many cell layers. This stage is associated with the production of extracellular factors, including the polysaccharide intercellular adhesion component of the extracellular matrix

Pseudomonas aeuroginosa

Pseudomonas aeruginosa is a gram-negative bacterium found in almost every ecological niche, including soil, water, and plants. It is also an important opportunistic pathogen of humans, primarily infecting immuno-compromised patients (Govan and Deretic, 1996). Recent reports indicate that environmental and clinical *P. aeruginosa* strains are functionally equivalent and taxonomically indistinguishable (Fought et al., 1996). The success of *P. aeruginosa* in various environments is attributed to its broad metabolic versatility and its elaboration of many cell-associated and secreted virulence/survival factors.

Bacteria in natural habitats usually grow as biofilms, organized communities of cells embedded in an extracellular polysaccharide matrix and attached to a surface (Costerton et al., 1995)

ENZYMES FOR BIOFILM CONTROL

Monitoring and control of biofilms accumulation remains the challenging to many industries. Previous studies have indicated that antimicrobial agents such as chemical biocides were the main strategy to control and prevent the formation of biofilm (Walker *et al.,* 2007). In many industries it is important that both the inactivation and the removal of biofilms from the surfaces are achieved (Simoes *et al.,* 2003). A wide range of biocides have been used in controlling biofilms, moreover these cleaning chemical agents have little to no effect at removing an established biofilms (walker *et al.,* 2007). Therefore application of enzymes would be an attractive strategy for the control and removal of biofilms. Enzymes remove biofilm by destroying the physical integrity of the biofilm matrix (EPS) (Xavier et al., 2005). Study material Study made by Loiselle *et al,* (2003) indicated that cellulases from *Penicillium funiculusum* was one of the most effective enzymes in degrading mature biofilms of *Pseudomonas aeruginosa*. Cellulases were also found to be effective in degrading the exopolysaccharides from *Pseudomonas fluorescens (*Loiselle et al., 2003; Vickery et al., 2004*).* Wiatr(1991) tested five enzymes in the biofilm removal reactor(BRR) and among those enzymes was a combination of one protease and two carbohydrates, namely alpha- amylase and beta-glucanase and the enzymatic mixture was found to be effective in digesting slime layers produced by cultures of pure and mixed strains of bacteria.

WHY ENZYMES?

Promoting detachment is the least investigated of the possible strategies to remove unwanted biofilms (Stewart et al., 2000). The use of substances to induce biofilm removal directly by destroying the physical integrity of the biofilm matrix would be an attractive alternative for both medical and industrial applications where complete biofilm removal is essential. In industrial applications, this approach would also have the advantage of reducing reliance on inherently toxic antimicrobial agents, whose continued use is fundamentally at odds with the trend towards increasingly restrictive environmental regulations (Chen & Stewart, 2000).

This question can be more easily answered by looking at the structure and composition of the biofilm as has been discussed before. Biofilms are primarily composed of bacteria, extracellular

Polymeric substances (EPSs) of microbial origin and other Abbreviations: DPA, detachment-promoting agent; EPS, extracellular polymeric substance; particulate substances. Biofilm EPS's are typically composed of diverse substances, including polysaccharides, proteins, nucleic acids, lipids and humid substances (e.g. Nielsen et al., 1996; Tsuneda et al., 2003). Previous studies have indicated that disinfection with chlorine dioxide and chlorine, for example, can reduce the concentrations of planktonic bacteria, but have little to no effect on the concentrations of biofilm bacteria (Berry et al., 2006). The mechanism behind the resistance of biofilms to disinfection is through protection of the biofilm cells that are embedded in the extracellular polymeric substances (Xavier et al., 2005; Walker et al., 2007). Enzymes have been used and proven to be effective for the degradation of the multi-structural EPS of the biofilms (Johansen et al., 1997; Melo et al., 1997; Augustin et al., 2004; Lequette et al., 2010). The mode in which enzymes destroy the EPS is by degrading the physical integrity of the EPS (Xavier et al., 2005). Walker et al. (2007) indicated that in order to design enzymes that target the EPS of the biofilms, it is important to have an understanding of the nature of the EPS. The efficiency of any one enzyme degrading EPS will depend on the EPS composition (Xavier et al., 2005; Walker et al.2007).

Previous studies have been published regarding enzyme degradation of mature biofilms using synthetic polysaccharides (Loiselle et al., 2003; Vickery et al., 2004). Cellulase from *Penicillium funiculusum* was effective in degrading mature biofilms of *Pseudomonas aeruginosa*; and it was also found to be useful in degrading the exopolysaccharides of *Pseudomonas fluorescens* (Loiselle et al., 2003; Vickery et al., 2004). Therefore, the application of enzymes to degrade EPS is a promising and an attractive option in many industries where complete biofilm removal is essential.

Table 1 properties of the commercial enzymes tested in this study

			Optima conditions		
Enzyme	**Manufacturer**	**Source**	**pH**	**Temperature(˚C)**	**Application**
Cellulase	SRL	*Aspergillus niger*	5	37	Food, feed and brewing industry
α-Amylase	SD Fine Chem ltd.	*Aspergillus niger*	4.5	25	Fermentation and pharmaceutical industries

Pectinase	SRL	*Aspergillus niger*	4	25	Brewery, industrial wastewater treatment

ABOUT THE ENZYMES

CELLULASE

Cellulase is a term used to describe a diverse class of hydrolases produced chiefly by fungi, bacteria, protozoans, and termites, catalysing the hydrolysis of cellulose (Lee et al., 2000; Watanabe et al., 1998). Cellulases are of great importance as they can be used to biodegrade cellulose containing biomass. Cellulases are helping not only in the removal of agricultural biomass that is continuously accumulating in the environment, but also simultaneously earning of monetary benefits from effective utilization of waste.

Cellulases are a highly diverse suite of enzymes that catalyze a single common reaction, i.e. the hydrolysis of the β-1, 4 bonds that join many of the two glucose molecules in a cellulose molecule. Cellulases are quite different from most enzymes, because they have the ability of degrading an insoluble substrate. For this to happen the enzyme diffuses to the substrate, followed by the movement a segment of a cellulose molecule from the insoluble particle into its active site, on the contrary the soluble substrates simply diffuse to the enzyme and bind into the active site by themselves (Wilson, 2008).

Even though there is a vast amount of knowledge about cellulase structure and function, the details of the mechanism by which these enzymes cause the degradation of the complex structure of their insoluble substrate i.e. Cellulose, remains unclear. Knowledge on the enzymatic hydrolysis of cellulose published nowadays has changed little compared to that postulated decades ago; making it clear that not much progress has been made in this field. Utilizing the wealth of knowledge about the structure and the catalytic activity of cellulolytic enzymes into the mode of their action on the insoluble substrate has been difficult because of the heterogeneous morphology of the cellulosic substrate and lack of strategies to overcome the difficulties in

visualizing the action of cellulases on the cellulose surface at the nanometer scale (Bubner et al, 2012)

AMYLASE

The starch degrading enzyme amylase is widely distributed in nature amylases are one of most important and widely used enzymes whose spectrum of application has widen in many sectors such as clinical, medicinal and analytical chemistry. Beside their use in starch saccharification they also find applications in food, baking, brewing, detergent, textile and paper industries. Amylases cover a class of industrially utilized enzymes having approximately 25% of the enzyme market share. An extra-cellular amylase, specifically starch digesting raw amylases has an important application in bioconversion of starches and starch-based substrates. The amount of activity alpha amylases that is present in human body fluid is of a very high clinical importance e.g. in diabetes, pancreatitis and cancer research whereas the alpha amylase present in plant and microbial are used as industrial enzymes.

Alpha Amylase's official name is 1, 4-a-D-Glucan glucanohydrolase. It is an enzyme which helps in the dissociation of starch to maltose. It hydrolyzes bonds between glucose repeats. Alpha amylase breaks down starch by hydrolysis to release maltose. It is the major form of amylase found in humans and other mammals. The α-amylases are calcium metalloenzymes, which are unable to function in the absence of calcium, α-amylase breaks down long-chain carbohydrates by acting at random locations along the starch chain, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. .The alpha-amylase can neither cleave terminal glucose residues nor α-1, 6-linkages. The final products of α-amylase action are oligosaccharides with different length with an α-configuration and α-limit dextrins, which constitute a mixture of maltose, maltotriose, and branched oligosaccharides of 6–8 glucose units that contain both α-1, 4 and α-1, 6 linkages.

Amylases together with cellulases are important enzymes for global carbon cycle on Earth. They are capable of degrading starch and related polymers. However due to bond specificity of these enzymes, complete and efficient degradation of starch usually requires the action of many different enzymes.

PECTINASE

Pectinases are classified into polysaccharide hydrolases, polysaccharide lysase and carbohydrate esterases based on the mechanism used to attack the galacturonan backbone and these include endo-polygalacturonases, exo-polygalacturonases, pectate lyases, pectin lyases and pectin methyl esterases. Endo-polygalacturonase is responsible to catalyse hydrolysis of α-1, 4 glycosidic linkages among the two non-methylated acidic residues. Endo-polygalacturonase are mainly produced by bacteria, fungi and yeast. Exo-polygalacturonase are mainly glycoprotein and there are two types of exo polygalacturonase which cleave α-(1, 4) glycosidic bonds of GAlA residues. Pectin lyases are responsible for degradation of pectin polymer by β-elimination mechanism, whereas pectin methyl esterases are responsible for catalyze the esterification mechanism of pectin. Pectin lyases are also catalyzing the trans-eliminative cleavage of the galacturonic acid polymer.

WORK PLAN

MICROTITER PLATE BIOFILM ASSAY

MATERIALS Bacterial strains *Pseudomonas aeuroginosa, Staphylococcus aureus*, *Escherichia coli* in Appropriate media for the strain (AIM-LB Broth w/o Trace Element(Himedia), 70% ethanol ,0.1% (w/v) crystal violet, for staining and a 96-well microtiter plates, not tissue culture treated with lids for growing the culture , spectrophotometer for adjusting the absorbance of our cultures and microscope for the visualization of our stained biofilms

METHOD The strains were inoculated in a 5-ml media and which was then grow to different phases in bacterial growth curve. Cultures were dilute in the medium to obtain the right desired growth phase which was ensured by measuring the optical density of the culture. 100 µl of each diluted culture was pipeted into each wells in a fresh microtiter . The plates were covered and incubated at optimal growth temperature overnight or 16-18 hrs. Planktonic bacteria was removed from each microtiter dish by through washing using distilled water. 125 µl of 0.1%

crystal violet solution was added to each well and kept for 10 min at room temperature.the plates were washed again to remove thes excess stain.This step removes any crystal violet that is not specifically staining the adherent bacteria.Invert each microtiter dish and vigorously tap on paper towels to remove any excess liquid. Allow the plates to air-dry. Visualize the stained culture under microscope.

GROWTH CURVE ANALYSIS

It is performed to obtain the difference in the growth pattern between two cultures placed under a single varying condition for cell growth. Two flask containing 100 ml media were inoculated with the culture (*S. aureus).* These cultures were then incubated at 37˚C under shaking and non shaking conditions. The absorbance, at 600 nm, for both these cultures was monitored with the gap of an hour and the results thus obtained were plotted onto a graph to establish the rough estimate for the time at which the biofilm starts developing.

TREATMENT OF BIOFILM WITH ENZYMES

QUALITATIVE ASSAY:

The strains were inoculated in a 3-to-5-ml culture and allowed to grow till OD 0.8. The culture was then diluted by adding medium to obtain the desired OD. 100 µl of each diluted culture was pipette into each well in a fresh microtiter plate. The plates were then covered and incubated at optimal growth temperature overnight or 16-18 hrs. The cultures were washed to remove the planktonic cells from the culture. The culture in the plate was incubated with enzymes of varying concentration and incubated for 2 hrs at 37˚C without shaking.

Each well was soaked for 1 min in saline (0.9%, w/v), before drying. then stained for 2 min in crystal violet followed by their visualization.

QUANTITATIVE ASSAY:

Cultured strains were transferred to the micro titer plate as mentioned before. These cultures were then incubated with the varying concentration of enzymes and the data was read on BIORAD 680 microplate reader to obtain the minimum concentration of the enzyme that was

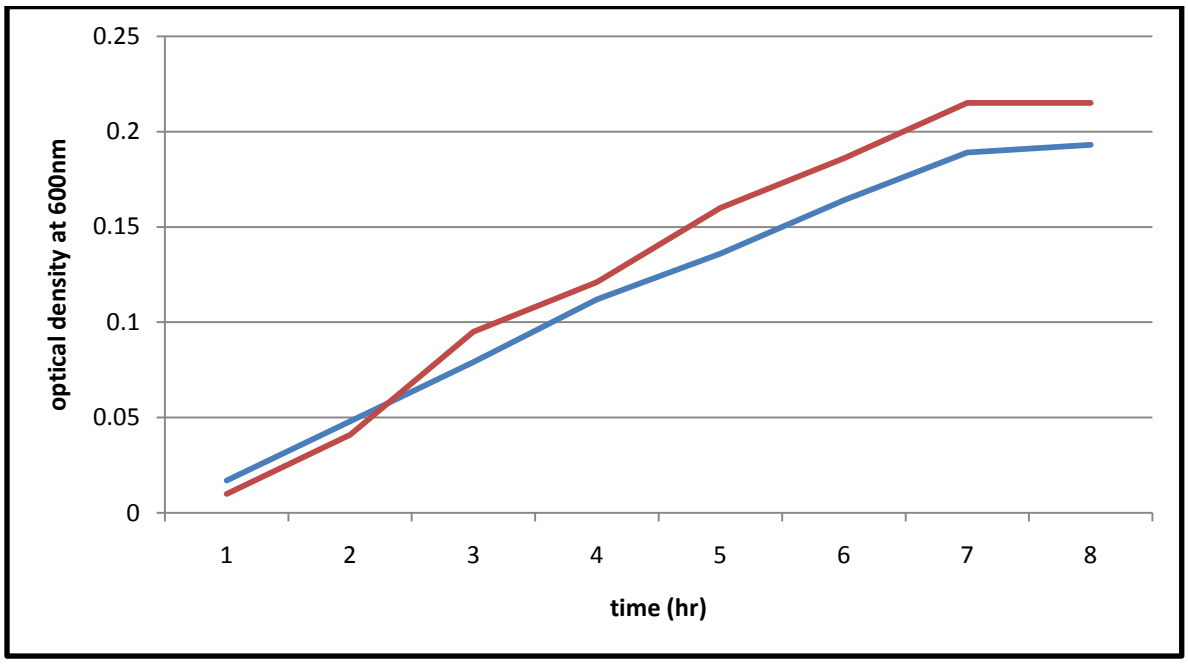
able to cause maximum degradation of the bacterial biofilm. Once that was obtained, the culture was then incubated with that very enzyme concentration for varying period of time to obtain the minimum time required.

RESULTS AND DISCUSSIONS

Using the microtiter plate assay is an effective way to monitor bacterial attachment to an abiotic surface over time. In general, if this assay were performed over several time points, one would expect to see a progressive increase, possibly followed by a decrease, in attached biomass. The eventual decrease in crystal violet staining is presumed to occur because the lack of nutrients may stimulate the bacteria to detach from the surface. The staining pattern also varies from one organism to another. For example, when *P. aeruginosa* is grown and stained with crystal violet the entire well shows positive crystal violet staining.

GROWTH CURVE ANALYSIS

The red line in the following graph represents the growth curve for the culture kept under non-shaking condition and the blue line is for the culture incubated under shaking conditions. The culture incubated under non-shaking conditions displayed a greater absorbance as compared to the other one as the stagnant environment helped the colonies to aggregate and develop slime layer thus increasing the overall density of the culture and hence the absorbance, whereas under shaking conditions the continuous agitation of the flask prevents the formation of cell aggregates in the media i.e., no biofilm formation and a comparatively low optical density.

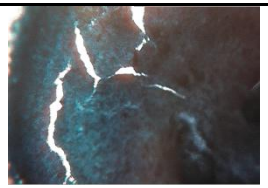
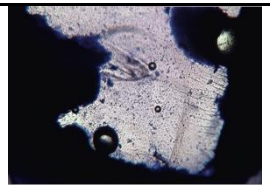
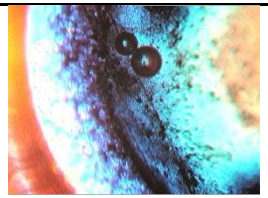
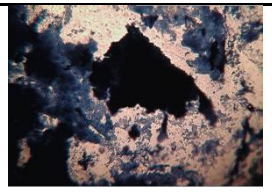
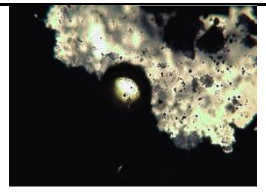
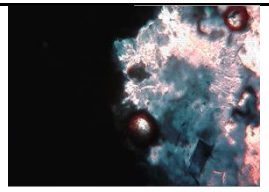


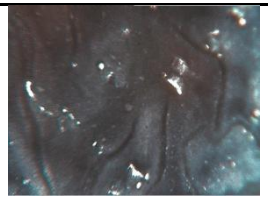
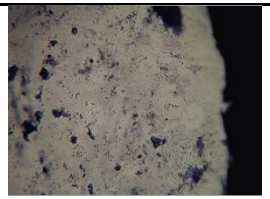
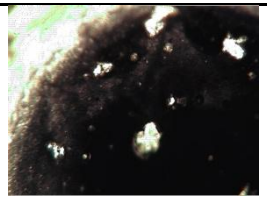
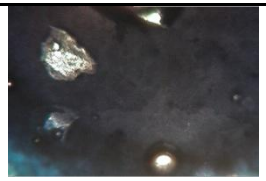
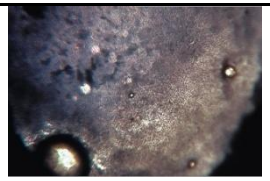
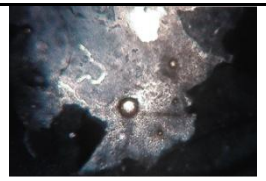
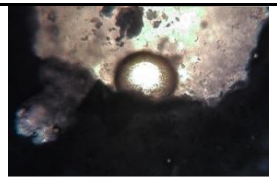
ENZYMATIC DEGRADATION OF BACTERIAL BIOFILM

QUALITATIVE ASSAY

Degradation of biofilm by enzymes followed by staining for visualization of the degraded region of the biofilm allows us to compare the action of enzymes on the biofilm by observing the end results.

Table 2 Qualitative comparison of the action of enzyme amylase used in varying concentration

Strain	Biofilm structure	After treatment with enzyme (alpha-amylase)		
		Enzyme concentration(U/mg)		
	Control	2000	200	20
S. aureus	 The stained image displays the untreated biofilm formed in the microtitre well plate	 The unstained/ lightly stained region here demonstrates the biofilm that has been removed after enzymatic action	 The unstained/ lightly stained region here demonstrates the biofilm that has been removed after enzymatic action	 The minimum concentration of enzyme here has given the least degradation of the biofilm
E. coli		 The biofilm formed by *E. coli* has mostly been removed expect for some flakes.	 Biofilm at the bottom of the well has been removed while it still is present on the side of the wall	 Biofilm at the bottom of the well has been removed with some flakes still remaining while it still is present on the side of the wall

P. aeroginosa		 Almost all of the biofilm formed in the well have been removed except for a few colonies	 Biofilm formed at the base have been removed while the area surrounding it still has biofilm in abundance	 Biofilm only removed in flakes
Mixed strain		 Partial degradation of the biofilm	 Partial degradation of biofilm	 Partial degradation of biofilm and non-removal from the surface of the wall

Staphylococcus aureus

The strain was moderately affected by the action of enzyme amylase in varying concentration. The biofilm formed at the bottom of the well was mostly removed while there were still traces of biofilm on walls of the well. With the decrease in the concentration of the enzyme the area of affected biofilm has also decreased depicting the decreased activity. Enzyme amylase has shown the maximum activity against the following strain at the minimum concentration of 2000 U/mg

Escherichia coli

The biofilm formed by this strain was mostly removed except for a few flakes that remained attached. Also we observed the same pattern of decreased activity of the enzyme amylase. The maximum activity was obtained at 2000 U/mg which was reduced as we reduced the concentration of the enzyme. Also as we moved down with enzyme concentration, a decrease in the degradation of the biofilm present on the walls of the well was also observed.

Pseudomonas aeuroginosa

The biofilm formed from this strain was most sensitive to the action of the enzyme. Most of biofilm was removed with only a few colonies remaining that can be seen in the picture as the stained dots. A maximum degradation was observed at the enzyme concentration of 2000 U/mg closely followed by the concentration 200 U/mg.

The 20 U/mg of the enzyme concentration though showed degradation in a very minute quantity as only a few flakes from the surface of the biofilm formation were removed.

MIXED STRAIN

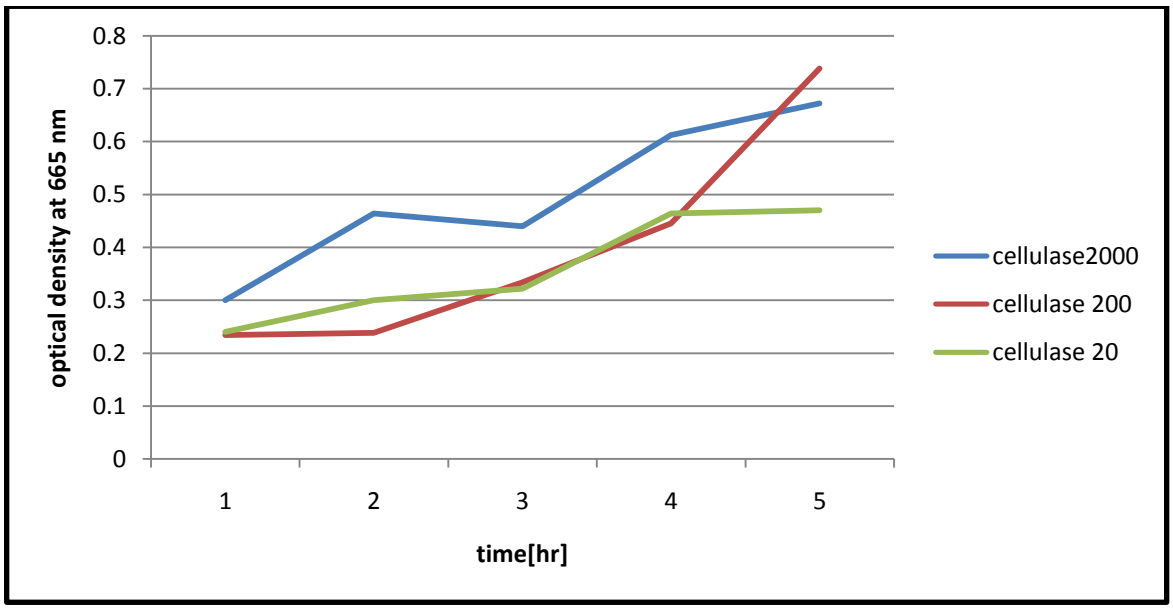
The concoction made up of the above mentioned strains was the most resistant towards the enzyme activity. Only partial removal of the biofilm from the surface of the well was observed. The lightly stained region depicts the layer of the biofilm that enzyme was unable to remove which can be observed in all the three concentration of the enzyme used.

QUANTITAIVE ASSAY

Once the elimination of bacterial biofilm was obtained by the use of enzymes of choice and visualized for degradation, the method was then optimized for determining the minimum concentration of the enzyme required. Also the combinations of enzymes that can be used for complete removal of the biofilm growth from the culture as the heterogeneity of the extracellular polysaccharides in the biofilm.

CELLULASE

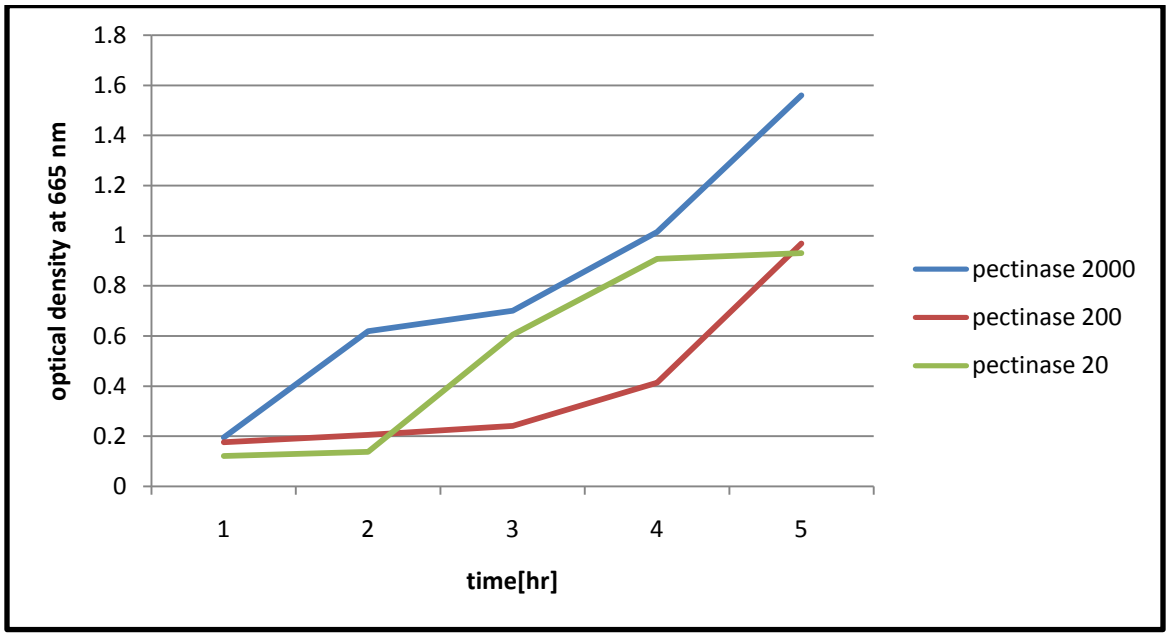
Cellulase on incubation with bacterial strains gives maximum degradation at the concentration of both 2000 U/mg and 200 U/mg as can be seen in the graph. The minimum concentration thus required of cellulose is 200 U/gm. On performing the time series analysis for the same concentration we obtained the following graph wherein the minimum time for the minimum concentration is 5 hrs.



PECTINASE

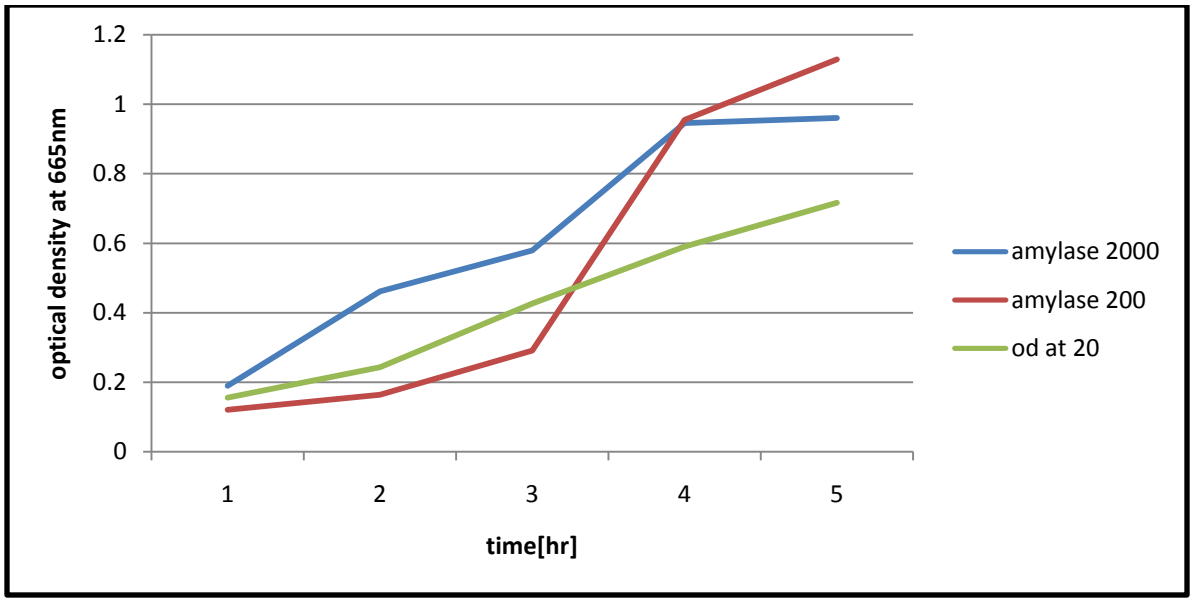
Pectinase enzyme in varying concentration was incubated with the bacterial biofilm and it demonstrated maximum degradation of the biofilm at 2000 U/mg (blue line) and the time series

analysis presented us with the graph with minimum time required for maximum degradation coming out to be 2 hrs and above



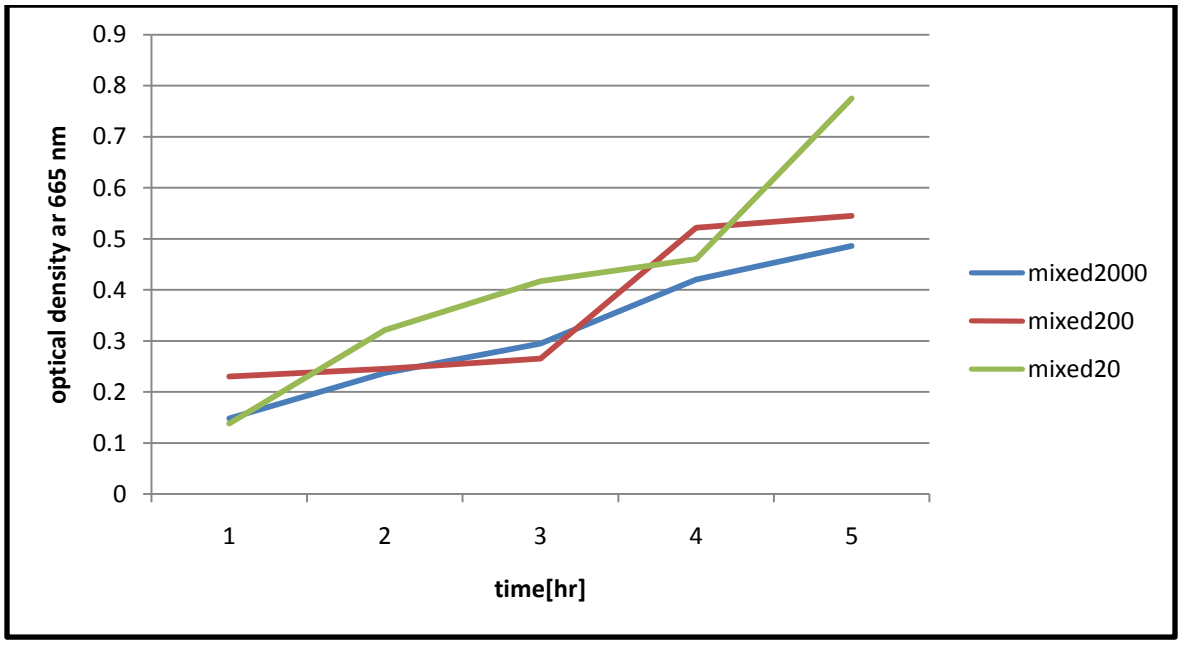
AMYLASE

Amylase enzyme was incubated with the bacterial strains for both qualitative and quantitative estimation. Results for qualitative estimation have been discussed before while the quantitative estimation gave us the minimum concentration required i.e. 2000 U/mg, closely followed by 200 U/mg. The minimum time taken by the minimum concentration (2000 U/mg) is also represented in the graph.



MIXED ENZYMES

All three above mentioned enzymes having the same activity were mixed together in the same amount. This solution was then used to react with the bacterial biofilm. The minimum concentration of this solution that displayed the maximum biofilm eradication was 20 U/mg followed by 200 U/mg. The time series analysis presented us with the following graph with increasing level of eradication with every one hour giving maximum degradation at 5 hrs.



CONCLUSION

The growth curve analysis confirms the fact that slime layer formation occurs mostly in a stagnant form of the culture. The culture in non-shaking condition demonstrated comparatively higher absorbance that is indicative of the aggregate formation in the culture. If this culture is allowed to sit for longer duration a clearly visible layer on top of the surface of the culture and along the walls of the container is formed. This layer is classified as biofilm and the conclusive reason for the higher absorbance.

The qualitative and the quantitative assay, both clearly show the degrading activity of the enzymes and by comparing that we can conclude that a single enzyme is insufficient for complete detachment of the biofilm. A concoction of enzymes in optimized quantity and for particular time duration is more effective in eradicating biofilm. The further research can be focused on reducing the amount of enzyme required for enzyme degradation along with the time that will produce better results and reduce the efforts put in the physical and chemical methods for biofilm eradication.

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