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ISOLATION AND IDENTIFICATION OF BIOFILM FORMING BACTERIA BASED ON BIOCHEMICAL TESTS AND ITS APPLICATION TOWARDS BIOREMEDIATION

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Abstract

Bio films are the thick dense masses of bacterial or algal cells that are commonly found on the stagnant water bodies or moist localities. Most of the biofilms are proved to be beneficial to the environment and are ecofriendly. These biofilms are responsible for the bioremediation of its substrate thereby maintaining the pollution free environment. The current work aims to isolate and identify the normal habitat of biofilm, once the identification is performed than the same bacteria can be employed in bioremediation process. The work involves the isolation of bacterial cultures from the test biofilm. Identification of the cultures using Bergey's manual this is followed by 16s RDNA sequencing to confirm the bacteria. The molecular techniques like DNA isolation using routine laboratory protocols, gene amplification using PCR and Gel electrophoresis to identify the genome of the isolated strain are employed. The work also involves specific tests to screen the bioremediation efficacy of the isolated species.

1. Introduction

The development of industry causes serious changes in the composition of water, soil, and air, which in turn leads to the breach in the ecological balance. The swift growth of human population and chemical industries over the past few decades has resulted in contamination of the environment due to toxic waste (Paul, D. et al., 2005). The persistence of chemical pollutants and consequent environmental problems has brought

the possibility of long-term environmental disasters into the public conscience (Pandey, G. et al., 2002). This problem is topical for the whole world, determining the need to introduce cleaner technologies and more effective treatment of waste. It leads to a policy on the control status of components and environmental factors. Therefore, various strategies are being developed and further research is currently underway to develop means of sustaining the environment (Yotova, et al., 2012).

Bioremediation is an emerging in situ innovative technology that controls the contamination. It uses biological systems, which catalyze the degradation or transformation of various toxic chemicals. In recent years a major role in bioremediation is of biofilms (Yotova, et al., 2012). The potential of biofilm communities for bioremediation n process has recently been realized. Biofilm-mediated bioremediation presents a proficient and safer alternative to bioremediation with microorganisms because cells in a biofilm have a better chance of adaptation and survival (especially during periods of stress) as they are protected within the matrix (Decho, A.W. 2000). Owing to the close, mutually beneficial physical and physiological interactions among organisms in biofilms, the usage of xenobiotics is accelerated and, consequently, biofilms are used in industrial plants to help in immobilization and degradation of pollutants.

Biofilms are clusters of microbial cells that are attached to a surface. They occur in nearly every moist environment where sufficient nutrient flow is available and surface attachment can be achieved. A biofilm can be formed by a single bacterial species, although they can also consist of many species of bacteria, fungi, algae and protozoa. Approximately 97% of the biofilm matrix is either water, which is bound to the capsules of microbial cells, or solvent, the physical properties of which (such as viscosity) are determined by the solutes dissolved in it (Sutherland, I.W. 2001). Growing importance in the economy, health and ecology on the one hand and the complex structure on the other led to intense interdisciplinary research on biofilms with participation of specialists from different fields: biotechnologists, microbiologists, chemists, engineers, physicists and others. Biofilms are used in industry and ecology. Biofilms are effectively applied in wastewater treatment, industrial water and air pollution and others. Although most chemical nature have an inhibitory effect on the growth of micro organisms is proved that biofilms formed by different strains are able to overcome this

effect and to degrade the contaminants using them as carbon source (Sgountzos, I.N. et al., 2006).

The present study is carried out in the laboratory to isolate and identify the biofilm forming bacteria based on biochemical tests. The study is further focused on to observe the role of the isolated bacteria in bioremediation of plastic waste.

2. Objective

The main objective of this study aims to identify the Bacteria that would be the normal habitat of the pollution prone areas and capable of causing bioremediation of plastic wastes. The work involves the application of the molecular techniques like DNA isolation using routine laboratory protocols, gene amplification using PCR and Gel electrophoresis to identify the genome of the isolated strain so as to preserve the DNA sample and extend the research in mere future and also to screen the bioremediation efficacy of the isolated species.

3. Proposed Methodology

3.1 Biofilms

The discovery of microorganisms, 1684, is usually ascribed to Antoni van Leeuwenhoek, who was the first person to publish microscopic observations of bacteria (Madigan MT, et al., 2003**)**. Although the most common mode of growth for microorganisms on earth is in surface associated communities (Sutherland, I.W. 2001), the first reported findings of microorganisms “attached in layers” were not made until the 1940s. During the 1960s and 70s the research on “microbial slimes” accelerated but the term “biofilm” was not unanimous formulated until 1984 (Bryers JD. 2000). Various definitions of the term biofilm have been proposed over the years. According to the omniscient encyclopedia Wikipedia a biofilm is “a structured community of microorganisms encapsulated within a self-developed polymeric matrix and adherent to a living or inert surface” (http://en.wikipedia.org, 20090205). Dental plaque, surfaces of slippery stones and pebble in a stream, slimy coatings in showers or on boat hulls, gunge on infected wounds or the mass clogging water distribution pipes are examples

of biofilms that may be encountered in ones everyday life. Microorganisms in biofilms produce extracellular polymeric substances (EPS) that hold the cell aggregates together and form the structural biofilm matrix scaffold (Branda SS, 2005). The fact that EPS is produced even under growth-limiting conditions, despite the high energy consumption it requires, emphasizes the advantages for bacterial cells to be in biofilm (Castonguay MH, et al., 2006). The biofilm matrix shelters the bacterial cells from antimicrobial agents and environmental stress by acting as a physical barrier (Davey, M.E. 2000). Other ecological advantages of the biofilm lifestyle are metabolic cooperation, presence of microniches and facilitated gene transfer. Efficient metabolic cooperation or mutual dependence (syntrophism) frequently evolves within biofilms due to interspecies substrate exchange facilitated by the spatial proximity of the cells. Development of microniches with diverse oxygen and nutrient concentrations within biofilms creates favorable conditions for a great variety of species. Enhanced gene transfer rates, often detected in biofilm communities, guarantees a progressive evolution and genetic diversity increasing the competitiveness of the bacterial cells (Davey, M.E. 2000). Bacterial cells adapted to a surface-associated lifestyle express phenotypic traits distinct from those expressed during planktonic growth. For example, increased tolerance to antimicrobial agents, altered metabolic or biochemical reaction rates, enhanced degradation ability of toxic chemicals and changed synthesis of biomolecules have been observed. Biofilms were initially thought of as homogenous systems of cells entrapped in slime but recent research findings point in the opposite direction. Nowadays, the perception of physiologic and genetic heterogeneity in biofilms is generally accepted in the research community (Wimpenny J, et al., 2000). Natural biofilms usually harbour a multitude of microbial species forming complex differentiated populations capable of developing highly convoluted structures, often separated by a network of water channels (Kolter R, et al., 2006). This requires a sophisticated organization which in some organisms is controlled by a cell-cell communication system, known as quorum sensing. The biofilm structure is also affected by numerous other conditions, such as surface and interface properties, nutrient availability, microbial community composition and hydrodynamics (Davey, M.E. 2000).

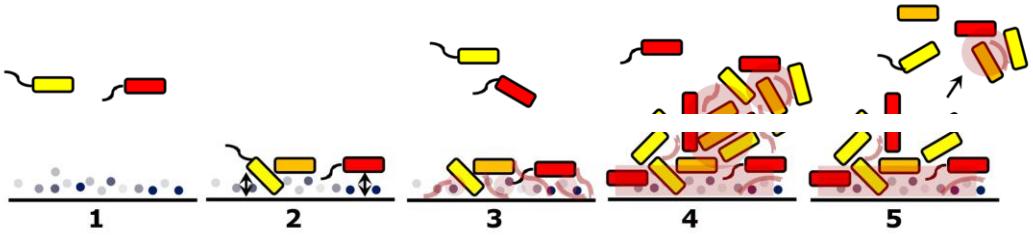
3.1.2 Biofilm formation and development

Biofilm formation and development is a fascinatingly intricate process, involving altered genetic genotype expression, physiology and signal molecule induced

communication. Biofilms can form on all types of surfaces, biotic or abiotic, in most moist environments. Several distinct steps essential in the biofilm formation process have been identified and a simplified sketch of the most crucial ones can be seen in Figure 5. Surfaces in aquatic environments generally attain a conditioning film of adsorbed inorganic solutes and organic molecules (Figure 5-1). Bacteria move towards the surface by chemotaxis or Brownian motion, resulting in a temporary bacteria-surface association (Figure 5-2) mediated by non-specific interactive forces such as Van der Waals forces, electrostatic forces, hydrogen bonding, and Brownian motion forces (Gottenbos B, et al., 1999). At the surface, production of extracellular polymeric substances will firmly anchor the cells to the surface. This state is commonly referred to as irreversible attachment (Figure 5-3), truly irreversible only in the absence of physical or chemical stress. Synthesis of exopolysaccharides which form complexes with the surface material and/or secretion of specific protein adhesins that mediate molecular binding are known mechanisms for irreversible attachment (Dunne WM, Jr., 2002). A large group of such proteinaceous adhesins are the b-sheet-rich, water insoluble amyloid fibrils found in 5-40% of the strains present in both freshwater and wastewater treatment biofilms (Larsen P, et al., 2007). During the initial attachment various short range forces are involved, including covalent, hydrogen and ionic bonding as well as hydrophobic interactions. The initially adhered cells rarely come in direct contact with the surface because of repulsive electrostatic forces; instead the secreted polymers link the cells to the surface substratum (Kumar CG, et al., 1998). The shift from reversible to irreversible attachment is relatively rapid. Various studies report firm attachment within a few minutes or less (Palmer J, et al., 2007). Once anchored at the surface, cell division and recruitment of planktonic bacteria results in growth and development of the biofilm community, i.e. maturation (Figure5-4).

Surface attached bacterial cells use the nutrients in the conditioning film and the aqueous bulk to grow and produce more EPS resulting in the formation of microcolonies. Eventually the microcolonies expand to form a layer covering the surface (Kumar CG, et al., 1998). During biofilm growth a differentiation of the gene expression pattern can be seen compared to planktonic cells. The production of surface appendages involved in bacterial motility is down-regulated due to cell immobility in the biofilm matrix while production of EPS and membrane transport proteins such as porins is up-regulated. The up- and down-regulation of genes is mainly dependent on

population density and is controlled by a signal molecule driven communication system known as quorum sensing (Dunne WM, Jr., 2002).



Schematic representation of the steps involved in biofilm formation.

1. Formation of conditioning film on the surface, 2. Initial adherence of bacterial cells, 3. Irreversible attachment of bacteria, 4. Maturation of the biofilm, 5. Detachment.

Mature bacterial biofilms are dynamic, spatially and temporally heterogeneous communities which can adopt various architectures depending on the characteristics of the surrounding environment (nutrient availability, pH, temperature, shear forces, osmolarity) as well as the composition of the microbial consortia (De Lancey, et al., 2001). Complex structures such as mushroom-like towers surrounded by highly permeable water channels, facilitating the transport of nutrient and oxygen to the interior of the biofilms, are commonly observed. The biofilm development process is fairly slow; several days are often required to reach structural maturity. A mature biofilm is a vibrant construction, with an advanced organization which continuously adapts itself to the surroundings, meaning that under adverse conditions bacteria may leave their sheltered existence within the biofilm community in the search for a new, more favorable habitat to settle down in. This step is known as detachment (Figure 5-5). The biological, chemical, and physical factors that drive detachment are complex. Degradation of the extracellular polymeric substances, absence of sufficient nutrients or oxygen, quorum sensing, hydraulic shear and normal forces, sloughing and erosion are all

factors believed to influence biofilm detachment (Chambless JD, et al., 2007). Active detachment involves an up regulation of genes encoding carbohydrate degrading enzymes resulting in weakened cohesive forces within the biofilm and subsequent detachment of single cells or biofilm units. Simultaneously the expression of porin proteins is down-regulated and the operon encoding flagella proteins is up-regulated, preparing the cells for a planktonic lifestyle (Sutherland, I.W. 2001).

3.1.3 Spreading of Biofilms in Nature

Biofilms exist everywhere. Almost all organisms, not only bacteria and archaebacteria have mechanisms that can attach to surfaces and each other. Biofilms can be found on rocks, pebbles, and often on the surface of stagnant pools. In fact, biofilms are an important component of the food chain in the rivers and the streams consumed by some rivers invertebrates, which in turn are a food for many fishes (Paul, D. et al., 2005).

Biofilms may be useful, constructive purpose. For example, many businesses sewage purification include a phase in which contaminated water passes over the biofilms placed on the filters ad so nutrients are extracted and used by the microorganisms of the films. In such water treatment bacteria are mainly responsible for removal of organic matter, while protozoa and Rotatoria are responsible for the removal of suspended salts also pathogenic and other microorganisms. Sand filters rely on biofilm development for the same reason, the purification of water from lakes, springs or the sources for drinking water (Belkin, Sh. 2003).

3.1.4 Extracellular Polymeric substances (EPS)

Biofilms retains its integrity and is protected by a matrix composed of microorganisms isolated from polymeric compounds called extracellular polymeric substances (EPS). Other compounds in the composition of the matrix are extracellular polysaccharides, proteins, uronic acids, nucleic acids, lipids and others. This matrix protects biofilm cells, enhances and facilitates relationships between them through biochemical signals. There are open water channels in some biofilms that help the distribution of nutrients among the biofilm cells. Extracellular polymeric substances are high molecular mass polymers with a molecular mass between 500 and 200kDa. They can be associated with one another, interact with other components of matrix itself, as proteins, lipids, ions and other macro molecules from the bacterial cell surface. So they can form a polymer network, which determines viscosity of the biofilm state. The extracellular matrix varies widely in composition, structure and properties and it is impossible to summarize its contribution to the formation and properties of the biofilms (Dreeszen, P.H. 2003)

3.1.5 Genetic and Environmental Regulation of biofilm formation

Various environmental factors regulate bacterial biofilm formation such as variations in pH, availability of nutrients and oxygen and concentration of bacterial metabolites, which cause biofilms to differ from each other so that a biofilm formed in a stream

differs from one formed on biological tissue. This fact was also substantiated by Allegrucci et al. 2006, who observed that environmental factors affect de novo protein production, within biofilms in Streptococcus pneumoniae;

simultaneously, an increase in cell number and biomass was also observed. Quorum sensing (the ability of bacteria to communicate and coordinate behavior through signaling molecules) also regulates changes in mature biofilms by controlling the formation of channels and pillar-like structures that ensure efficient nutrient delivery to the cells. This architecture helps in the adequate dispersal of nutrients when there is increased competition for food at high cell density within biofilms (Kjelleberg, S. et al., 2002).

To gain an insight into the genes involved in biofilm formation, DNA microarrays representing >99% of the annotated Bacillus subtilis open reading frames were used to follow temporal changes in gene expression that occurred during the transition from the planktonic to biofilm state. Approximately 6% of the genes involved in motility, phage-related functions, metabolism and transcription of B. subtilis were differentially expressed (Stanley, N.R. et al. 2003). The transcription factor RpoS affected biofilm formation during different phases in Pseudomonas aeruginosa and Escherichia coli as indicated by microarray studies. Whiteley et al. 2001 also demonstrated that certain subpopulations in a biofilm exhibit a different pattern of gene expression than the planktonic cells or other metabolically active cells. Lazazzera, B.A. 2005 reported the repression of flagellar genes and hyperexpression of genes responsible for adhesion and formation of ribosomal proteins at various stages of biofilm formation. In E. coli biofilms, ribosomal genes were expressed at an increased level when compared with planktonic cells in stationary phase but not in exponential phase (Schembri, M.A. et al. 2003). Therefore, various microorganisms show differences in their gene expression patterns during biofilm formation.

3.1.6 Role of biofilms in bioremediation

Successful application of a bioremediation process relies upon an understanding of interactions among microorganisms, organic contaminants and soil or aquifer materials. Physiological properties of the microorganisms such as biosurfactant production and chemotaxis enhance bioavailability and, hence, degradation of hydrophobic compounds (Pandey, G. et al., 2002). Microorganisms that secrete polymers and form biofilms on the surface of hydrocarbons are especially well suited for the treatment of recalcitrant or slow degrading compounds because of their high microbial biomass and ability to

immobilize compounds by biosorption (passive sequestration by interactions with biological matter), bioaccumulation (increased accumulation of microbes under influence) and biomineralization (formation of insoluble precipitates by interactions with microbial metabolic products) (Barkay, T. et al., 2001). Biofilms support a high biomass density that facilitates the mineralization processes by maintaining optimal conditions of pH, localized solute concentrations and redox potential in the vicinity of the cells. This is achieved by the unique architecture of the biofilm and controlled circulation of fluids within it (Flemming, H.C. 1995). Biofilm-based reactors are commonly used for treating large volumes of dilute aqueous solutions such as industrial and municipal wastewaters. The main biofilm reactors are categorized according to the methods they use, such as the upflow sludge blanket (USB), biofilm fluidized bed (BFB), expanded granular sludge blanket (EGSB), biofilm airlift suspension (BAS) and internal circulation (IC) methods. In USB, BFB and EGSB reactors the particles are kept fluidized by an upward liquid flow. In BAS reactors, a suspension is obtained by pumping air into the system, whereas in IC reactors, the gas produced in the system drives the circulation and mixing of the liquid and solids in an airlift-like reactor (Nicolella, C. et al. 2000).

3.2 Bioremediation

Bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminant compounds are transformed by living organisms through reactions that take place as a part of their metabolic processes. Biodegradation of a compound is often a result of the actions of multiple organisms. When microorganisms are imported to a contaminated site to enhance degradation we have a process known as bioaugmentation. For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. As bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate.

Bioremediation techniques are typically more economical than traditional methods such as incineration, and some pollutants can be treated on site, thus reducing exposure

risks for clean-up personnel, or potentially wider exposure as a result of transportation accidents. Since bioremediation is based on natural attenuation the public considers it more acceptable than other technologies. Most bioremediation systems are run under aerobic conditions, but running a system under anaerobic
conditions (Colberg, et al., 1995) may permit microbial organisms to degrade otherwise recalcitrant molecules.

3.2.1Microbial Populations for Bioremediation Processes

Microorganisms can be isolated from almost any environmental conditions. Microbes will adapt and grow at subzero temperatures, as well as extreme heat, desert conditions, in water, with an excess of oxygen, and in anaerobic conditions, with the presence of hazardous compounds or on any waste stream. The main requirements are an energy source and a carbon source. Because of the adaptability of microbes and other biological systems, these can be used to degrade or remediate environmental hazards. We can subdivide these microorganisms into the following groups:

Aerobic (In the presence of oxygen). Examples of aerobic bacteria recognized for their degradative abilities are *Pseudomonas*, *Alcaligenes*, *Sphingomonas*, *Rhodococcus*, and *Mycobacterium*. These microbes have often been reported to degrade pesticides and hydrocarbons, both alkanes and polyaromatic compounds. Many of these bacteria use the contaminant as the sole source of carbon and energy.

*Anaerobic (*In the absence of oxygen). Anaerobic bacteria are not as frequently used as aerobic bacteria. There is an increasing interest in anaerobic bacteria used for bioremediation of polychlorinated biphenyls (PCBs) in river sediments, dechlorination of the solvent trichloroethylene (TCE), and chloroform.

Ligninolytic fungi. Fungi such as the white rot fungus *Phanaerochaete chrysosporium* have the ability to degrade an extremely diverse range of persistent or toxic environmental pollutants. Common substrates used include straw, saw dust, or corn cobs.

Methylotrophs. Aerobic bacteria that grow utilizing methane for carbon and energy. The initial enzyme in the pathway for aerobic degradation, methane monooxygenase, has a broad substrate range and is active against a wide range of compounds, including the chlorinated aliphatics trichloroethylene and 1,2-dichloroethane (Barkay, T. et al., 2001).

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3.3 Bioremediation Strategies

Different techniques are employed depending on the degree of saturation and aeration of an area. *In situ* techniques are defined as those that are applied to soil and groundwater at the site with minimal disturbance. *Ex situ* techniques are those that are applied to soil and groundwater at the site which has been removed from the site via excavation (soil) or pumping (water). *Bioaugmentation* techniques involve the addition of microorganisms with the ability to degrade pollutants.

3.3.1 In situ bioremediation

These techniques are generally the most desirable options due to lower cost and fewer disturbances since they provide the treatment in place avoiding excavation and transport of contaminants. *In situ* treatment is limited by the depth of the soil that can be effectively treated. In many soils effective oxygen diffusion for desirable rates of bioremediation extend to a range of only a few centimeters to about 30 cm into the soil, although depths of 60 cm and greater have been effectively treated in some cases.

The most important land treatments are:

Bioventing is the most common *in situ* treatment and involves supplying air and nutrients through wells to contaminated soil to stimulate the indigenous bacteria. Bioventing employs low air flow rates and provides only the amount of oxygen necessary for the biodegradation while minimizing volatilization and release of contaminants to the atmosphere. It works for simple hydrocarbons and can be used where the contamination is deep under the surface.

In situ biodegradation involves supplying oxygen and nutrients by circulating aqueous solutions through contaminated soils to stimulate naturally occurring bacteria to degrade organic contaminants. It can be used for soil and groundwater. Generally, this technique includes conditions such as the infiltration of water-containing nutrients and oxygen or other electron acceptors for groundwater treatment.

Biosparging. Biosparging involves the injection of air under pressure below the water table to increase groundwater oxygen concentrations and enhance the rate of biological degradation of contaminants by naturally occurring bacteria. Biosparging increases the mixing in the saturated zone and thereby increases the contact between soil and groundwater. The ease and low cost of installing small-diameter air injection points allows considerable flexibility in the design and construction of the system.

Bioaugmentation. Bioremediation frequently involves the addition of microorganisms indigenous or exogenous to the contaminated sites. Two factors limit the use of added microbial cultures in a land treatment unit: 1) nonindigenous cultures rarely compete well enough with an indigenous population to develop and sustain useful population levels and 2) most soils with long-term exposure to biodegradable waste have indigenous microorganisms that are effective degrades if the land treatment unit is well managed (Kolter R, et al., 2006).

3.3.2 Ex situ bioremediation

These techniques involve the excavation or removal of contaminated soil from ground. *Landfarming* is a simple technique in which contaminated soil is excavated and spread over a prepared bed and periodically tilled until pollutants are degraded. The goal is to stimulate indigenous biodegradative microorganisms and facilitate their aerobic degradation of contaminants. In general, the practice is limited to the treatment of superficial 10–35 cm of soil. Since landfarming has the potential

T o reduce monitoring and maintenance costs, as well as clean-up liabilities, it has received much attention as a disposal alternative.

Composting is a technique that involves combining contaminated soil with nonhazardous organic amendants such as manure or agricultural wastes. The presence of these organic materials supports the development of a rich microbial population and elevated temperature characteristic of composting.

Biopiles are a hybrid of landfarming and composting. Essentially, engineered cells are constructed as aerated composted piles. Typically used for treatment of surface contamination with petroleum hydrocarbons they are a refined version of landfarming that tend to control physical losses of the contaminants by leaching and volatilization. Biopiles provide a favorable environment for indigenous aerobic and anaerobic microorganisms (Dunne WM, Jr., 2002).

Bioreactors. Slurry reactors or aqueous reactors are used for *ex situ* treatment of contaminated soil and water pumped up from a contaminated plume. Bioremediation in reactors involves the processing of contaminated solid material (soil, sediment, sludge) or water through an engineered containment system. A slurry bioreactor may be defined as a containment vessel and apparatus used to create a three-phase (solid, liquid, and gas) mixing condition to increase the bioremediation rate of soil bound and water-soluble pollutants as a water slurry of the contaminated soil and biomass (usually

indigenous microorganisms) capable of degrading target contaminants. In general, the rate and extent of biodegradation are greater in a bioreactor system than *in situ* or in solid-phase systems because the contained environment is more manageable and hence more controllable and predictable. Despite the advantages of reactor systems, there are some disadvantages. The contaminated soil requires pre treatment (e.g., excavation) or alternatively the contaminant can be stripped from the soil via soil washing or physical extraction (e.g., vacuum extraction) before being placed in a bioreactor.

3.4 Bioremediation and biofilm communities

Biofilm-mediated bioremediation presents a proficient and safer alternative to bioremediation with planktonic microorganisms because cells in a biofilm have a better chance of adaptation and survival (especially during periods of stress) as they are protected within the matrix (Decho, A.W. 2000). Owing to the close, mutually beneficial physical and physiological interactions among organisms in biofilms, the usage of xenobiotics is accelerated and, consequently, biofilms are used in industrial plants to help in immobilization and degradation of pollutants. Akinson reported the use of biofilms for water and wastewater treatments in the early 1980s. However, it is only during the past few decades that biofilm reactors have become a focus of interest for researchers in the field of bioremediation.

Numerous studies have determined the composition of communities present in biofilms in various environments (Tani, K. et al. 1995) and a growing number of microscopic and molecular methods have facilitated the analysis of spatial organization and phylogenetic properties of microbes in a biofilm community (Chalfie, M. et al. 1994). Growing importance in the economy, health and ecology on the one hand and the complex structure on the other led to intense interdisciplinary research on biofilms with participation of specialists from different fields: biotechnologists, microbiologists, chemists, engineers, physicists and others. Biofilms are used in industry and ecology. Biofilms are effectively applied in wastewater treatment, industrial water and air pollution and others. Although most chemical nature have an inhibitory effect on the growth of micro organisms is proved that biofilms formed by different strains are able to overcome this effect and to degrade the contaminants using them as carbon source (Sgountzos, I.N. et al., 2006).

3.5 Molecular approach to study microbial communities

3.5.1 Analyses of naturally ocurring rRNA and rDNA

The starting point for the molecular approach and related procedures is the extraction of nucleic acids of sufficient quality to permit activity of the enzymes used in subsequent procedures, as Polymerase Chain Reaction (PCR). This is not a trivial matter and it has been one of the main challenges in our working group as discussed above.

There are two strategies based on rRNA and rDNA to identify bacteria in sample material. The first approach is based on the recovery of rRNA that is transcribed into cDNA, cloned and sequenced (Ward *et al*., 1990). The alternative approach is based on the recovery of high molecular weight DNA directly from sample material, followed by the amplification of rDNA by PCR (Polymerase Chain Reaction), cloning and sequencing. The result of both strategies is a clone library, containing ribosomal sequences as inserts.

Agarose gel electrophoresis is a method used in DNA fingerprinting to separate DNA, or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix under the influence of an electric field. The agarose is having high net like structure. Shorter molecules moves faster and cover more distance. The samples can also be recovered integrity of the nucleic acid can be established.

Agarose is extract from red algae (sea weed) and linear polymer of diffrent from from of galactose. Agarose gel can be casted by melting the agarose in the presence of desired buffer until a clear, transparent solution is obtained. The melted solution is the poured in to a mold and allowed to solidify. Upon hardening, agarose forms a matrix the density of which is determined by concentration of the agarose . When electric charge is applied across the gel DNA which is negatively charged at neutral pH migrates towards the anode.

The most common dye used to make DNA or RAN bands visible for agarose gel electrophoresis is ethidium bromide, usually abbreviated as EtBr. It fluoresces under the UV light when intercalated into DNA. It can absorb light at particular wavelength in the visible spectrum .BY running DNA through EtBr –treated gel and visualized it with UV light , distinict bands of DNA becomes visible . EtBr is known carcinogen and should be handled carefully.

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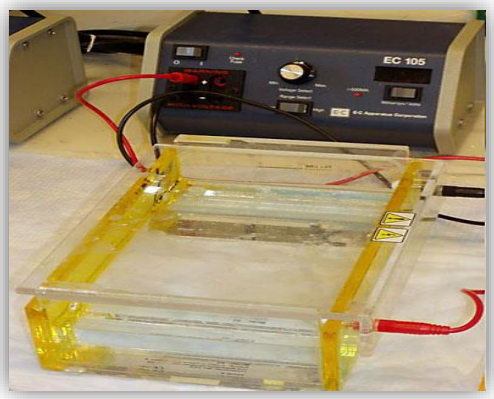

Electrophoretic unit

Factors affecting the gel electrophoresis

Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the log10 of their molecular. in other words if you plot the distance from the well that DNA fragments have migrated against the log10 of either their molecular weight or number base pairs, a roughly straight line will appear, several additional factors have important effects on the motility of DNA fragments in agarose gels, and can be used to your advantage in optimizing separation of DNA fragments chief among these factors are:

Agarose concentration: by using gel with different concentration of agarose one can resolve different sizes of DNA fragments. Higher concentration of agarose facilitates separation of small DNAs while lo agarose concentration allows resolution of larger DNAs.

Voltage: Ss the voltage applied to a gel is increased larger fragments migrate proportionally faster than small fragments.

Electrophoresis buffer: several different buffers have been recommended for electrophoresis of DNA, the most commonly used for duplex DNA are TAE (Tris-acetate EDTA) and TBE (tris-borate-EDTA) . DNA fragments will migrate at somewhat different rates in these two buffers is due to differences in ionic concentration.

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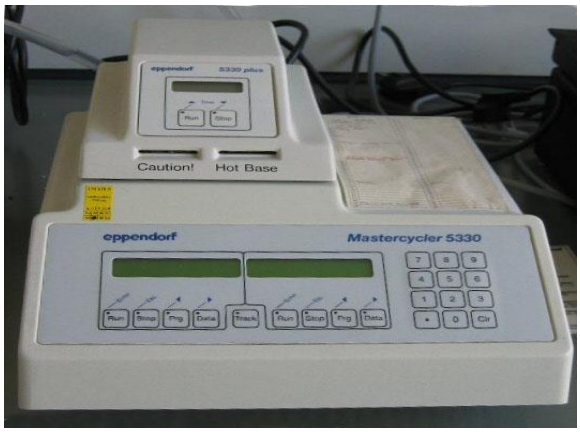
3.5.2 The PCR-clone-sequence approach

The extracted DNA is subjected to PCR amplification using "universal" primers or primers designed to amplify rRNA genes from particular group of organisms. The broad-range amplification of 16S rDNA genes with universal 16S rDNA primers allows the unselective detection of unexpected or hitherto unknown bacteria in medical and environmental samples. The use of specific primers for a specific group of microorganisms can be both highly specific and sensitive. The PCR products can then be cloned by overhanging 3' deoxyadenosine residues and blunt end ligation procedures, or by using commercially available kits for the cloning of PCR products.

Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.



Thermal Cycler

3.5.3 Sequencing of specific clones

Automated DNA sequencing systems have greatly facilitated the rapid screening and analysis of large gene libraries. Initial screening of rRNA gene-containing clones by different methods such as restriction fragments length polymorphism (RFLP) analysis of purified plasmid DNA or insert DNA obtained by colony PCR for the presence of near identical sequences, can greatly reduce the number of clones that require complete sequencing. However, RFLP is of limited use for demonstrating the presence of specific phylogenetic groups and is a time-consuming method. By sequencing individual clones

and comparing the obtained sequences with sequences present in databases, it is possible to identify the phylogenetic position of the corresponding bacteria without their cultivation.

3.5.4 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis (DGGE) is a method by which fragments of partial 16S rDNA amplified fragments of identical length but different sequence can be resolved electrophoretically due to their different melting behaviour in a gel system containing a gradient of denaturants. This method has been applied to the analysis of 16S rRNA genes from environmental samples (Muyzer *et al*., 1993). As a result, a band pattern is obtained, which reflects the complexity of the microbial community. By excising individual DGGE bands from the gel and reamplifying the DNA, it is possible to get sequence information of single community members

(Muyzer *et al.*, 1993; Smalla *et al.*, 1998). DGGE is relatively rapid to perform, and many samples can be run simultaneously. The method is, therefore, particularly useful when examining time series and population dynamics. Once the identity of an organism associated with any particular band has been determined, fluctuations in individual components of a microbial population, due to environmental perturbations, can be rapidly assessed. DGGE represents a powerful tool for monitoring microbial communities.

3.5.5 Whole-cell hybridization

This approach is Fluorescent *In Situ* Hybridization (FISH). End-labeled oligonucleotides are sufficiently sensitive to allow the specific detection of individual microbial cells directly in sample materials. Fluorescent rRNA-targeted oligonucleotide probes confer fluorescent stain specifically to cells of a phylogenetically coherent group on various taxonomic levels from species up to the kingdom level. They can be applied to samples without prior cultivation and determine the cell morphology and identity of microorganisms, their abundance and the spatial distribution *in situ* (Amann *et al.* 1995). Cells showing specific hybridization with the fluorochrome-labelled probe can be identified and enumerated. There are also some limitations associated with the technique. These can be divided in four main categories: cell permeability problems, target site accessibility, target site specificity and sensitivity.

3.6 Review of literature

Kae Kyoung Kwon, 2002 had isolated Bacterial strains from biofilms formed on glass slides submerged in seawater in Dae-Ho Dike. Eight strains showing fast attaching ability were selected and identified. Their exopolysaccharides (EPS)-producing ability and EPS properties were characterized. Based on Microlog System, 4 among the 8 strains were identified as *Micrococcus luteus* and the rest were *Bacillus thuringiensis, Bacillus megaterium, Staphylococcus saprophyticus* and *Agrobacterium vitis*. *A. vitis* was reidentified as *Sulfitobacter pontiacus* based on 16S rDNA sequence data. The amount of water-soluble EPS produced by the 8 strains ranged from 0.114 to 1.329 g·l-1 and the productivity was negatively correlated with the cell biomass. The molecular weight of the produced EPS ranged from 0.38 to 25.19´104 Da. Glucose and galactose were ubiquitous sugar components. Mannose, ribose, and xylose were also major sugar components. The molecular weight and composition of the EPS showed strain-specific variation**.**

Peacock, A.D. et al, 2003had observed the biofilms that formed on Bio-Sep beads had 2–13 times greater viable biomass; however, the bead communities were less metabolically active [higher cyclopropane/monoenoic phospholipid fatty acid (PLFA) ratios] and had a lower aerobic respiratory state (lower total respiratory quinone/ PLFA ratio and ubiquinone/menaquinone ratio) than the biofilms formed on glass wool. Anaerobic growth in these systems was characterized by plasmalogen phospholipids and was greater in the wells that received electron donor additions. Partial 16S rDNA sequences indicated that Geobacter and nitrate-reducing organisms were induced by the acetate, ethanol, or glucose additions. DNA and lipid biomarkers were extracted and recovered without the complications that commonly plague sediment samples due to the presence of clay or dissolved organic matter. Although microbial community composition in the groundwater or adjacent sediments may differ from those formed on down-well biofilm samplers, the metabolic activity responses of the biofilms to modifications in groundwater geochemistry record the responses of the microbial community to biostimulation while providing integrative sampling and ease of recovery for biomarker analysis.

Masaaki Morikawa, 2006 observed that biofilms are densely packed multicellular communities of microorganisms attached to a surface or interface. Bacteria seem to initiate biofilm formation in response to specific environmental cues, such as nutrient

and oxygen availability. Biofilms undergo dynamic changes during their transition from free-living organisms to sessile biofilm cells, including the specific production of secondary metabolites and a significant increase in the resistivity to biological, chemical, and physical assaults. Bacillus subtilis is an industrially important bacterium exhibiting developmental stages. It forms rough biofilms at the air-liquid interface rather than on the surface of a solid phase in a liquid, due to the aerotaxis of the cells. Biofilm formation by B. subtilis and related species permits the control of infection caused by plant pathogens, the reduction of mild steel corrosion, and the exploration of novel compounds. Although it is obviously important to control harmful biofilm formation, the exploitation of beneficial biofilms formed by such industrial bacteria may lead to a new biotechnology.

Dunne WM, 1992 has identified the effects of increasing concentrations of magnesium (Mg2+), calcium (Ca2+) or EDTA, and pH on the adhesion of five slime-positive strains of Staphylococcus epidermidis (Se+) to plastic were examined using an in vitro microwell assay. The addition of Mg2+ (as either MgSO4 or MgCl2) to the bacterial suspension in concentrations as low as 16 microM significantly enhanced the adhesion of all test strains to plastic ($P < 0.001$). Similarly, the addition of Ca2+ (as CaCl2) in concentrations exceeding 128 microM produced a significant increase in the adhesion of all test strains, but not to the extent observed with Mg2+. In contrast, the adhesion of all test strains to plastic was significantly reduced in the presence of EDTA at concentrations greater than 8 mM. However, EDTA in concentrations as low as 0.25 mM caused a significant decrease in the adhesion of two strains of Se+. The effect of pH was variable, but at a pH of 5.0 and 6.0, the adhesion of all test strains was significantly reduced compared to control values at a pH of 7.0. Two strains showed a significant increase in adhesion at a pH of 8.0. We also compared the effects of these variables on the adherence of a slime-negative phase variant derived from a slime-positive parent strain. With the exception of pH, the adhesion of both strains in response to increasing divalent cations or EDTA was similar. These data indicate that, in addition to hydrophobic interactions, ligand-specific binding, and slime production, pH and divalent cations, especially Mg2+, are important determinants of the adhesion of S. epidermidis to plastic surfaces in vitro.

Dunne WM, 1993 worked on the adhesion of five strains of slime-positive Staphylococcus epidermidis to plastic microwells was significantly diminished ($P <$

0.005) in a concentration-dependent fashion when wells were previously coated with increasing concentrations (1.6-13.1 micrograms cm-2) of human fibronectin (FN). The adhesion of four of five strains was significantly reduced when wells were coated with 3.2 micrograms cm-2 of FN and at concentrations > or = 6.5 micrograms cm-2 the adhesion of all slime-positive strains was significantly reduced. The coating of microwells with chymotryptic fragments of FN containing the heparin-binding, gelatin-binding, or cell-binding domains also reduced bacterial adhesion but none of the fragments exceeded the anti-adhesive activity of intact FN. A comparison of FN-coated or albumin-coated microwells showed that both proteins caused a significant reduction in the adhesion of test strains to plastic but that the anti-adhesive activity of FN was greater than albumin at all concentrations tested. The adhesion of the slime-negative phase variant of one of the test strains to plastic was neither enhanced nor reduced by FN coating indicating that the production of an exopolysaccharide by Staph. Epidermidis influences interactions with protein-coated surfaces. These results support the contention that FN does not mediate the adhesion of all strains of Staph. Epidermidis to plastic surfaces.

References

1. A.D. Peacock, Y.-J. Chang, J.D. Istok, L. Krumholz, R. Geyer, B. Kinsall, D. Watson, K.L. Sublette and D.C. White 2003. Utilization of Microbial Biofilms as Monitors of Bioremediation. Microbial ecology. 47, 284–292

2. Allegrucci, M. et al. (2006) Phenotypic characterization of Streptococcus

pneumoniae biofilm development. J. Bacteriol. 188, 2325–2335

3. Amann, R.I., W. Ludwig and K-H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143-169.

4. Barkay, T. and Schaefer, J. (2001) Metal and radionuclide bioremediation: issues, considerations and potentials. Curr. Opin. Microbiol. 4, 318–323

5. Belkin Sh. (2003). Microbial whole –cell sensing systems of environmental pollutants, Elsevier, Current Opinion Microbiology, 6, 203-212.

6. Branda SS, Vik S, Friedman L, and Kolter R, (2005), Biofilms: the matrix revisited. Trends Microbiol. 13(1): p. 20-26.

7. Bryers JD, (2000), Biofilms: an introduction, in Biofilms II: process analysis and applications, Bryers JD, Editor. Wiley-Liss: New York. p. 3-11.

8. Castonguay MH, et al., (2006), Biofilm formation by Escherichia coli is stimulated by synergistic interactions and co-adhesion mechanisms with adherence-proficient bacteria. Res Microbiol. 157(5): p. 471-478.

9. Chalfie, M. et al. (1994) Green fluorescent protein as a marker for gene expression. Science 263, 802–805

10. Chambless JD and Stewart PS, (2007), A three-dimensional computer model analysis of three hypothetical biofilm detachment mechanisms. Biotechnol Bioeng. 97(6): p. 1573-1584.

11. Davey ME and O'Toole GA, (2000), Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64(4): p. 847-867.

12. Decho, A.W. (2000) Microbial biofilms in intertidal systems: an overview. Cont. Shelf Res. 20, 1257–1273

13. De Lancey, Pulcini E, (2001), Bacterial Biofilms: a review of current research. Néphrologie. 22(8): p. 439-441.

14. Dreeszen, P.H. (2003). Biofilm, Edstorm Industries, INC., the key to understanding and controlling bacterial growth in Automated Drinking Water Systems, Second ed., 2003.

15. Dunne WM, Burd EM, 1992. The effects of magnesium, calcium, EDTA, and pH on the in vitro adhesion of Staphylococcus epidermidis to plastic. Microbiol Immunol. 36(10), 1019-27.

16. Dunne WM, Burd EM., 1993. Fibronectin and proteolytic fragments of fibronectin interfere with the adhesion of Staphylococcus epidermidis to plastic. J Appl Bacteriol. 74(4):411-6.

17. Dunne WM, Jr., (2002), Bacterial adhesion: seen any good biofilms lately? Clin Microbiol Rev. 15(2): p. 155-166.

18. Flemming, H.C. (1995) Sorption sites in biofilms. Water. Sci. Technol.32, 27–33

19. Gottenbos B, van der Mei HC, and Busscher HJ, (1999), Models for studying initial adhesion and surface growth in biofilm formation on surfaces. Methods Enzymol. 310: p. 523-534.

20. Kolter R and Greenberg EP, (2006), Microbial sciences: the superficial life of microbes. Nature. 441(7091): p. 300-302.

21. Kae Kyoung Kwon, Hyun Sang Lee, Sung-Young Jung, Joung-Han Yim, Jung-Hyun Lee, and Hong Kum Lee, 2002. Isolation and Identification of Biofilm-Forming Marine Bacteria on Glass Surfaces in Dae-Ho Dike, Korea. The Journal of Microbiology, 40 (4), p.260-266.

22. Kjelleberg, S. and Molin, S. (2002) Is there a role for quorum sensing

signals in bacterial biofilms? Curr. Opin. Microbiol. 5, 254–258

23. Kumar CG and Anand SK, (1998), Significance of microbial biofilms in food industry: a review. Int J Food Microbiol. 42(1-2): p. 9-27.

24. Larsen P, et al., (2007), Amyloid adhesins are abundant in natural biofilms. Environ Microbiol. 9: p. 3077-3090.

25. Lazazzera, B.A. (2005) Lessons from DNA microarray analysis: the gene expression profile of biofilms. Curr. Opin. Microbiol. 8, 222–227

26. Madigan MT, Martinko JM, and Parker J, (2003), Brock Biology of Microorganisms. 10 Ed. New Jersey: Pearson Education, Inc.

27. Masaaki Morikawa, 2006. Beneficial Biofilm Formation by Industrial Bacteria Bacillus subtilis and Related Species. Journal of bioscience and bioengineering, 101(1), 1–8.

28. Muyzer, G., E.C. de Waal and A.G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59:695-700.

29. Muyzer, G. and K. Smalla. 1998. Application of denaturing gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Anton. Leeuwenh. 73:127-141.

30. Nicolella, C. et al. (2000) Particle based biofilm reactor technology. Trends Biotechnol. 18, 312–320

31. Palmer J, Flint S, and Brooks J, (2007), Bacterial cell attachment, the beginning of a biofilm. J Ind Microbiol Biotechnol. 34(9): p. 577-588.

32. Pandey, G. and Jain, R.K. (2002). Bacterial chemotaxis toward environmental pollutants: role in bioremediation. Appl. Environ. Microbiol. 68, 5789–5795.

33. Paul, D. et al. (2005). Accessing microbial diversity for bioremediation and environmental restoration. Trends Biotechnol. 23, 135–142

34. P. J. S. Colberg and L. Y. Young. Anaerobic Degradation of Nonhalogenated Homocyclic Aromatic Compounds Coupled with Nitrate, Iron, or Sulfate Reduction. In Microbial Transformation and Degradation of Toxic Organic Chemicals, pp. 307–330, Wiley-Liss, New York (1995).

35. Schembri, M.A. et al. (2003) Global gene expression in Escherichia coli

biofilms. Mol. Microbiol. 48, 253–267

36. Stanley, N.R. et al. (2003) Identification of catabolite repression as a physiological regulator of biofilm formation by Bacillus subtilis by use of DNA microarrays. J. Bacteriol. 185, 1951–1957

37. Stoodley P, Sauer K, Davies DG, and Costerton JW, (2002), Biofilms as complex differentiated communities. Annu Rev Microbiol. 56: p. 187-209.

38. Sutherland, I.W. (2001). The biofilm matrix – an immobilized but dynamic microbial environment. Trends Microbiol. 9, 222–227.

39. Tani, K. et al. (1995) Development of a direct in situ PCR method for detection of specific bacteria in natural environments. Appl. Environ. Microbiol. 61, 4074–4082

40. Whiteley, M. et al. (2001) Gene expression in Pseudomonas aeruginosa

biofilms. Nature 413, 860–864

41. Wimpenny J, Manz W, and Szewzyk U, (2000), Heterogeneity in biofilms. FEMS Microbiol Rev. 24(5): p. 661-671.

42. Yotova L., Marinkova D., 2012. Investigation of the formation, structure and application of Biofilms (review): Journal of the University of Chemical Technology and Metallurgy, 47(3), 243-250.

43. Ward, D.M., R. Weller and M.M. Bateson. 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature. 345:63-65.