PROTEOMIC ANALYSIS OF BIOFILM AND PLANKTONIC CELLSS

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DEPARTMENT OF BIOTECHNOLOGY
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WAKNAGHAT

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CERTIFICATE

This is to certify that the work titled **" Proteomic Analysis Of Biofilm & Planktonic Cells"** submitted by **" Swati Sharma"** 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

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Date: 28/05/15

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Swati

SWATI SHARMA

SUMMARY

Cronobacter Sakazakii, a popular contaminan found in infant formula milk is a reason of causing necrotizing enterocolitis,bacteremia, and a rare form of infant meningitis.The gram-negative,rod- shaped bacteria of family Enterobacteriaceae induce signs and symptoms like poor feeding response, jaundice, variation in body temperature, hydrocephalus, developmental delay, and death.

Organism is ubiquitous in the water and soil environment and is also prevalent in food like dairy products. C.*Sakazakii* has been found to be resistant to antibiotics like vancomycin. Penicilin,oxacillin etc. generally a biofilm is composed of extracellular polymeric matrix. Like other bacteria. C*ronobacter* has known to show its persistance to food surfaces.A growing number of works have been and are publishing about the change in gene expression during biofilm formation that lead to its strong resistance In our work we have studied about the biofilm and planktonic culture through proteomic analysis by SDS gel running.

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 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 Hence analysing the proteomic structures involved in biofilm formation is the major goal of the project thereby understanding the major proteins that are contributing to such a resistant structure.

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INTRODUCTION

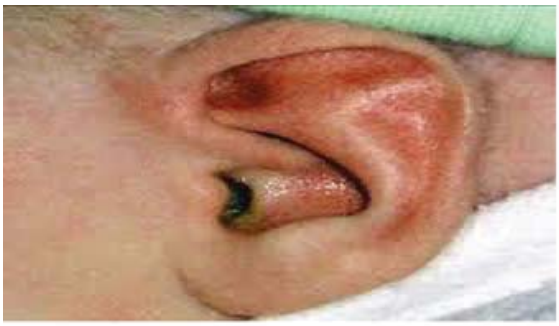
Microbial biofilms are known to play a crucial role in a variety of disciplines, including biotechnology, immunology, biofouling and biodeterioration . A biofilm is an aggregate of microorganisms in which cells stick to each other and or to a surface. These adherent cells are frequently embedded within 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.

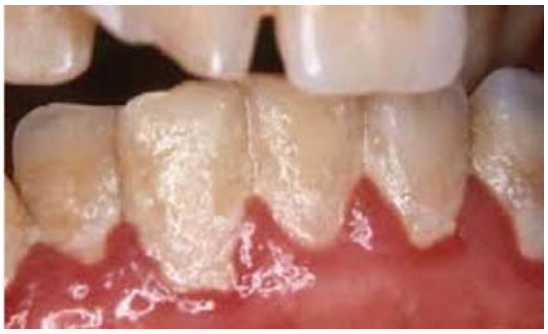
They are a common mode of bacterial growth in nature and their presence has an enormous impact on many aspects of our lives, such as sewage treatment, corrosion of materials, food contamination during processing, pipe collapse, plant-microorganisms interaction in the biosphere, the formation of dental plaque, the development of chronic infections in live tissue or problems related to medical implants.

Literature includes some evidence that cell contact with surfaces stimulates transcription of the EPS genes. Monitoring the EPS gene expression in adherent populations enables a better understanding on the basis of biofilm phenotype(Allison et al.,997; 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.

Biofilms have also been known to play a major role in causing a wide range of infections especially chronic infections. Infectious problems such as urinary infections, catheter infections, middle-ear infections, formation of dental plaque, gingivitis contact lenses, and may lead to more of lethal infections as cystic fibrosis, and infections of permanent indwelling devices such as joint prostheses and heart valves. More recently it has been noted that bacterial biofilms may impair cutaneous wound healing and reduce topical antibacterial efficiency in healing or treating infected skin wounds.

Biofilms can also be formed on the inert surfaces of implanted devices such as catheters, prosthetic cardiac valves and intrauterine devices.





INFECTIONS CAUSED BY BIOFILMS

(image source: www. drneal.com)

Over the past years, as scientists developed better tools to analyze 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.

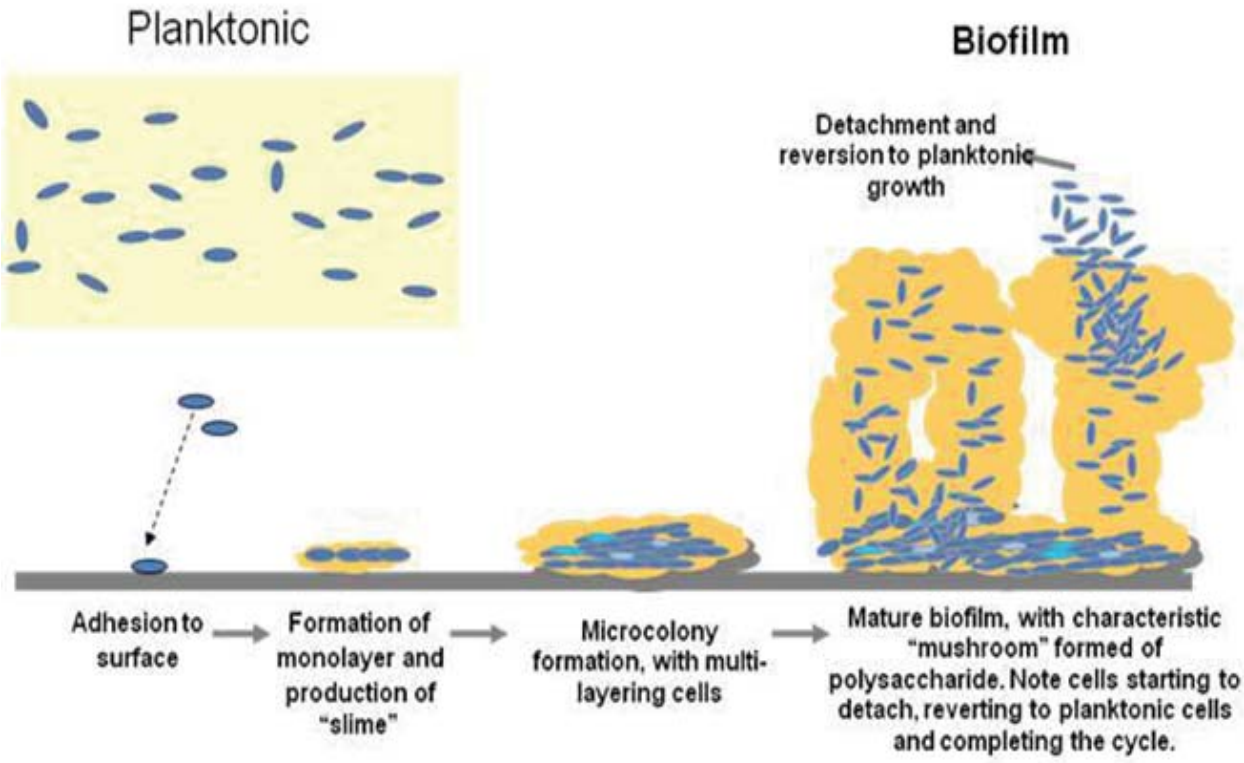
COMPOSITION OF EPS

As discussed earlier during biofilm formation bacterial cells secrete a sticky glue like material or slime as a result of which they are adhered or embedded in a thick layer termed as an extrapolysaccharide or EPS. EPS matrix thickness varies from bacterial species and is generally within 0.2 to 1.0 μm thick In some bacterial species, the thickness of the EPS layer does not exceed values from 10 to 30nm**.**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.
These microorganism features are often used during cell identification and classification procedures.

Most microbial exogenous layers contain neutral carbohydrates (mainly-hexose, seldom-pentose) and uronic acids. The commonest extracellular carbohydrates substituents are acetate esters, pyruvates, 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.

STAGES INVOLVED IN BIOFILM FORMATION

(IMAGE SOURCE: MEDCRAVEONLINE.COM)



1- PRIMARY STAGE

A primary stage is mainly an adhesion stage in which there is contact between the microorgansims and the surface Soon attachment process begins and, the organism is brought into close proximity of the surface, propelled either randomly or in a directed fashion via chemotaxis and motility . As soon as organism is attached to the surface.

The adhesion generally depends on net rate of attractive and repulsive forces. These forces include electrostatic and hydrophobic interactions and van der Waals attractions .This attachment is unstable and reversible and if the environment is not favorable for microbial attachment,cells can detach from the surface.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 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.When a biofilm is composed of heterogeneous species, the metabolic byproducts of one organism might serve to support the growth of another.

Adhesion of one species might provide ligands which allow the attachment of others. Conversely, the depletion of nutrients and accumulation of toxic byproducts generated by primary colonizers may limit the species diversity within a biofilm .A primary stage is mainly an adhesion stage in which there is contact between the microorgansims and the surface Soon attachment process begins and, the organism is brought into close proximity of the surface, propelled either randomly or in a directed fashion via chemotaxis and motility . As soon as organism is attached to the surface the adhesion generally depends on net rate of attractive and repulsive forces. These forces include electrostatic and hydrophobic interactions and van der Waals attractions .This attachment is unstable and reversible and if the environment is not favorable for microbial attachment,cells can detach from the surface.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 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.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. Conversely, the depletion of nutrients and accumulation of toxic byproducts generated by primary colonizers may limit the species.

2-SECONDARY STAGE

The secondary stage involves anchoring of bacteria to the surface by molecular mediated binding between specific adhesions and the surface. In this process loosely bound organisms gather together and produce exopolysaccharides that form a complex with surface materials 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 hence there is a change in a, gene expression.Hence at this stage there is increased amount of EPS production, increased resistance to antibiotics, increased UV resistance, resistance towards any type of disinfectant etc.

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 . They have distinct roles in different species and under different environmental conditions . 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 biofilm formation by overcoming repulsive forces. A number of aquatic bacteria possess fimbraie, which have also been shown to be involved in bacterial attachment.

3-MICROCOLONY FORMATION

After the adherence of bacteria to the inert surface, the association becomes stable for micro colony formation.The bacteria begin to multiply while sending out chemical signals that intercommunicate among the bactterial 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 .

Micro-colonies further develop into macro-colonies which are divided by fluid filled channels and enclosed in an extracellular polysaccharide matrix. 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.

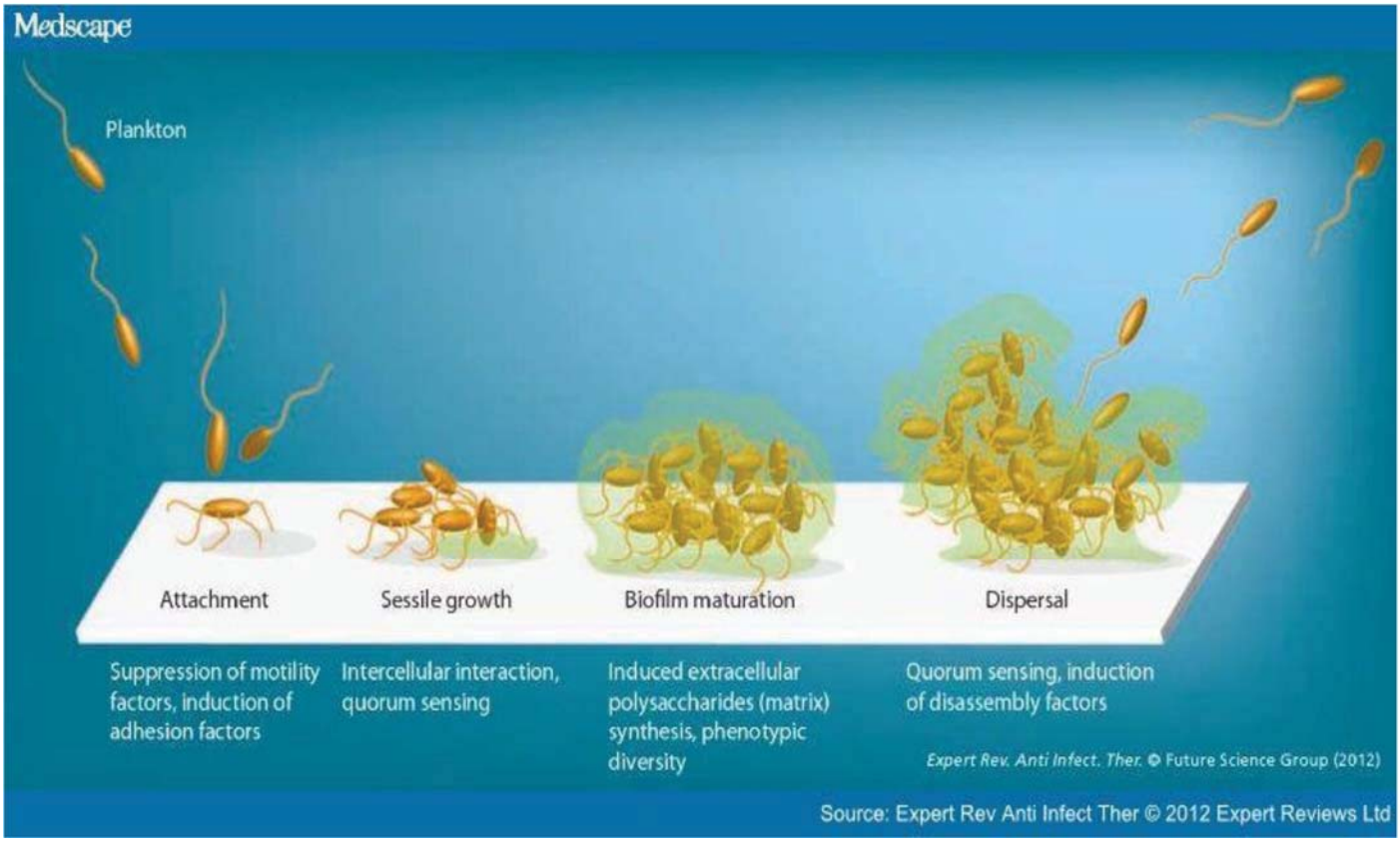
4) FORMATION OF THREE DIMENSIONAL STRUCTURE

During the attachment phase of biofilm development, the transcription of specific genes takes place. These are required for the synthesis of EPS . 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 microcolonies.

5) 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.

The availability of nutrients in the immediate environment within the biofilm and the removal of waste, limits the growth potential of any bacterial biofilm . In addition, there is an existence of an optimum hydrodynamic flow across the biofilm that determines the maximum growth. 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 or low surface energy materials that are either low positively or low negatively charged hydrophobic materials such as plastic made up of organic polymer 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 or increase in pH, O2 levels or an accumulation of toxic metabolic by products



BIOFILM MATURATION (image source:www. Medscape.com)

6) DETATCHMENT AND DISPERSAL

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.Some bacteria are shed from colony and some stop producing EPS and are released into the surrounding environment . Biofilm cells may be dispersed either by shedding of daughter cells from actively growing cells or detachment as a result of nutrient levels. The released microorganisms may be transported to new locations and restart the biofilm process

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 some studies, it was found that some species of bacteria species can synthesize polymer degrading enzymes to control the production of the EPS.

ABOUT THE STRAINS

Many strains been studied for their biofilm developingcharacteristics and proteomic analysis. In this project we have chosen to work on the following biofilm forming bacterial strains

(*E.coli* COLONY FORMATION)

1)*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 Outbreaks of *E. coli* infections have been primarily associated with eating undercooked ground beef, but a variety of other foods have also been implicated as vehiclesThe strain of *E.coli* which we are using is *DH5 Alpha E.coli* strain it's a non pathogenic strain and is mainly used in labs for cloning purposes which does not forms biofilm and has been used as a negative control.

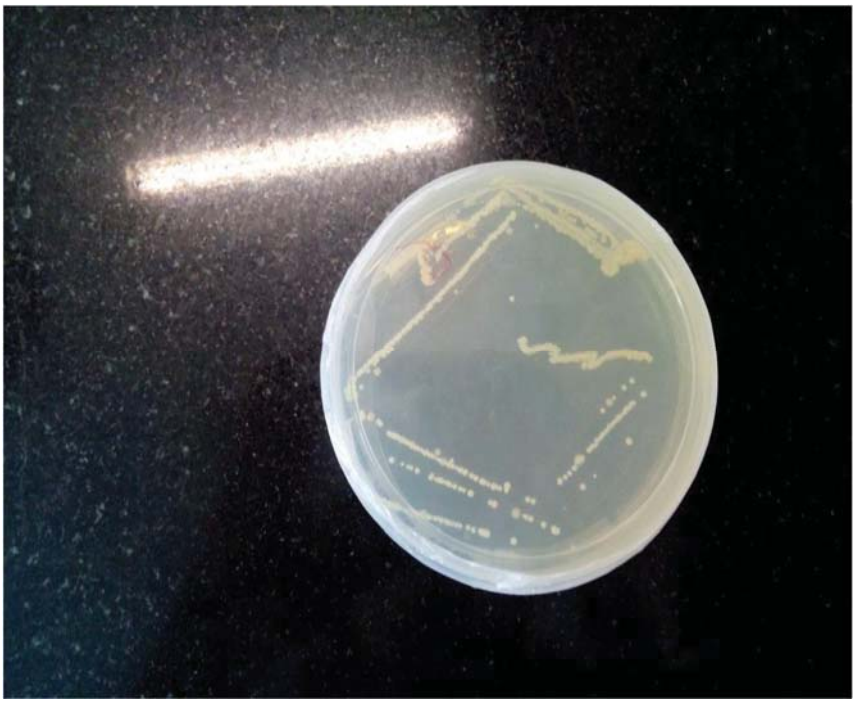
2) Cronobacter Sakazakii

The bacterium is ubiquitous being isolated from a range of environments and foods. *Cronobacter spp*. has been isolated in a various range of food, clinical, and environmental sources including meat, vegetables, grains, herbs, spices, stools, soils, and powdered infant formula (PIF). It is also found in food production facilities and households and has been detected at low levels in powdered infant formulas in a number of countries. Some strains are able to survive in a desiccated state for more than two years. However *Cronobacter* has been isolated from a wide range of foods and environmental and clinical sources, it is in association with intrinsically or extrinsically contaminated powdered formula which has attracted the main attention.

Cronobacter sakazakii is an opportunistic pathogen, affecting mainly newborns, and causes neonatal necrotizing enterocolitis and neonatal meningitis . Symptoms in newborns include fever, rapid heart rate, seizures and other neurological abnormalities.

Cronobacter sakazakii infections are often fatal in newborns. The death rate has been reported to be as high as 40-80% In older infants, children and adults, it can cause sepsis or respiratory illness Meningitis caused by *Cronobacter spp*. occurs both as sporadic cases and as outbreaks, and contaminated powdered infant formulas have been epidemiologically implicated as the source of the pathogen in most cases. In order to cause meningitis, it is expected that *Cronobacter spp*. express virulence factors that help in colonization of the mucosal surfaces, allow for the translocation into the bloodstream and overcome host defense mechanisms.

(*Cronobacter Sakazakii* –yellow colonies observed)



PROTEOMIC ANALYSIS

Since control of biofilms remains a major challenge to many industries like food industries. Hence it is important to know the virulent proteins that are involved in causing neonatal infections Farmer *et al.*,(1980) estabilished the taxonomic position of *Enterobacter Sakazakii* which was previously identified as yellow pigmented *Enterobacter Cloacae* using DNA -DNA hybridization techniques combined with phenotypic analysis.Schierack *et al*.,(2006)studied the virulence factor gene profiles of *E.coli* isolates from clinically healthy pigs.Isolation of *E.coli* was carried out from 34 pigs and 331 isolates were tested for genes encoding heat stable eneterotoxins and heat liable enterotoxins and atleast 68% of isolates were positive for atleast one virulent genes

Bhagat and Vridhi *et al*.,(2007) detected an array of virulence associated genes in *Cronobacter Sakazakii* genes through PCR amplification

Mullane *et al*.,(2008) investigated *Cronobacter* contamination from contaminated air filters in powdered milk process facility over a period of about 10 months. Seven air filters, environment and powdered products were analysed for presence of *Cronobacter Sakazakii* species.The effects of air filter installation,maintainence, and subsequent. dissemmination of *Cronobacter* were investigated. A total of 30 isolates were investigated by pulsed field electrophoresis that revealed the presence of three clonal populations

Hartmann *et al.,(*2010) investigated the genetic basis of biofilm formation in *Cronobacter* on abiotic surfaces by screening the library of random transposon mutants of *ES5* strain. *Jo et al*.,(2010) reported the maturation and survival of *Cronobacter* species in biofilms formed on silicone and polycarbonate and stainless steel immersed in powdered infant milk formulae even after UV light treatment and ethanol immersion.

Al-Nabulsi *et al.,(*2011) evaluated the effect of extreme Ph (3.5 for 30 minutes and 11.25 for 5 minutes),cold (4 degrees for 24 hrs),heat(55 degrees for 5 minutes) and dessication (cells were dried at 40 degree celcius for 2 hrs and held at 21 degree celcious for 4 days) stresses on suseptibilty of 5 isolated strain of *C.Sakazakii* on antibiotics such as-
streptomycin,gentamycin,kanamycin,neomycin,tetracyclin,vancomycin,etc.

Yan *et al*.,(2011) studied the inactivation of *Cronobacter* in dry infant formulae by UV treatment combined with hot water treatment at a temperature of around 55 degree to 60 degrees.Grim *et al*.,identified and characterized *Cronobacter* strains having iron accquisition systems with Feo and Efe systems responsible for transport of iron. Shin *et al*.,(2012) sequenced and analysed whole genome of *Cronobacter sakazakii ES1*.

WHY PROTEOMIC ANALYSIS?!

Bacterial communities that are attached to a surface, so-called biofilms, and their inherent resistance to antimicrobial agents are a major cause of many persistent and chronic bacterial infections. Recent genomic and proteomic studies have been identified & hence had been useful since many of the genes and gene products differentially expressed during biofilm formation have been identified, revealing the complexity of this developmental process. Biofilms have a distinct architecture, consisting of tower- and mushroom-shaped microcolonies encased in a hydrated matrix of exopolymeric substances, polysaccharides and proteins that are produced by the resident microorganisms. Compared with their planktonic (non-adherent) counterparts,the compact microbial consortia present in biofilms show extraordinary resistance to conventional biocides, antimicrobial treatments and the immune defense responses of the host.

Formation of these sessile communities and their inherent resistance to antimicrobial agents are at the root of many persistent and chronic bacterial infections. Biofilms have been shown to colonize a wide variety of medical devices and to be associated with several human diseases, such as native valve endocarditis, burn wound infections, chronic otitis media with effusion and cystic fibrosis. Recent advances in understanding of the genetic and molecular basis of bacterial community behavior point to therapeutic targets that may provide a means for the control of biofilm infections. Looking back, research on biofilms has come a long way since the initial characterization of a biofilm by Antoni Van Leeuwenhoek. The first descriptions of specific genes that are up- or down-regulated in biofilm bacteria were made using transcriptional *lacZ* reporter-gene fusions and led to the belief that bacterial attachment initiates the expression of a set of genes that culminates in a biofilm phenotype. That significant fractions of the bacterial genome could be involved in, or affected during, biofilm formation was shown in *Escherichia coli* in a genome-wide screen using random chromosomal insertions of a *lacZ* reporter gene fusion construct Prigent-Combaret *et al.* showed that bacteria within biofilms encounter higher-osmolarity conditions,greater oxygen limitation, and higher cell density than in the liquid phase with so many genes involved.

The availability of complete bacterial genome sequences, together with the development of microarrays with which the expression of the entire genome of an organism grown under two conditions can be assayed,has launched the post-genomic era of biofilm research and generated a wealth of new information. But a comparison of the differentially expressed gene sets identified in several recent DNA microarray studies reveals that no common expression pattern for biofilms has yet emerged. Instead, in different studies different genes are found up and down-regulated. For example, Gram-negative bacteria differ from Gram-positive with respect to cell-wall composition, the molecules involved in quorum sensing (the ability of bacteria to communicate with each other in a population to coordinate population behavior in response to environmental cues), and some transcriptional regulators and vary in level of protein expression too responsible for biofilm formation.

OBJECTIVES:

Hence the aims of the present proposed research are as follows-

1) Study their differential protein expression in planktonic & biofilm phase
2) Identification of Cronobacter biofilm proteins responsible for biofilm formation
3) Growth curve analysis

WORK PLAN

MICRITITRE PLATE BIOFILM ASSAY (A QUANTITATIVE ANALYSIS) - DETERMINATION OF GROWTH OF BIOFILM OR GROWTH CURVE ANALYSIS

MATERIALS-

1) Bacterial strains- *Cronobacter Sakazakii, E.coli* (being used as a negative control)
2) Growth media-Tryptic soy broth for *Cronobacter sakazakii* and LB broth for *E.coli*
3) Chemicals- 70% ethanol ,0.1% (w/v) crystal violet, for staining ,glacial acetic acid for suspension and methanol
4) Equipments- Spectrophotometer for adjusting the absorbance of our cultures, microtitre plates,pippete, tips

METHODS-

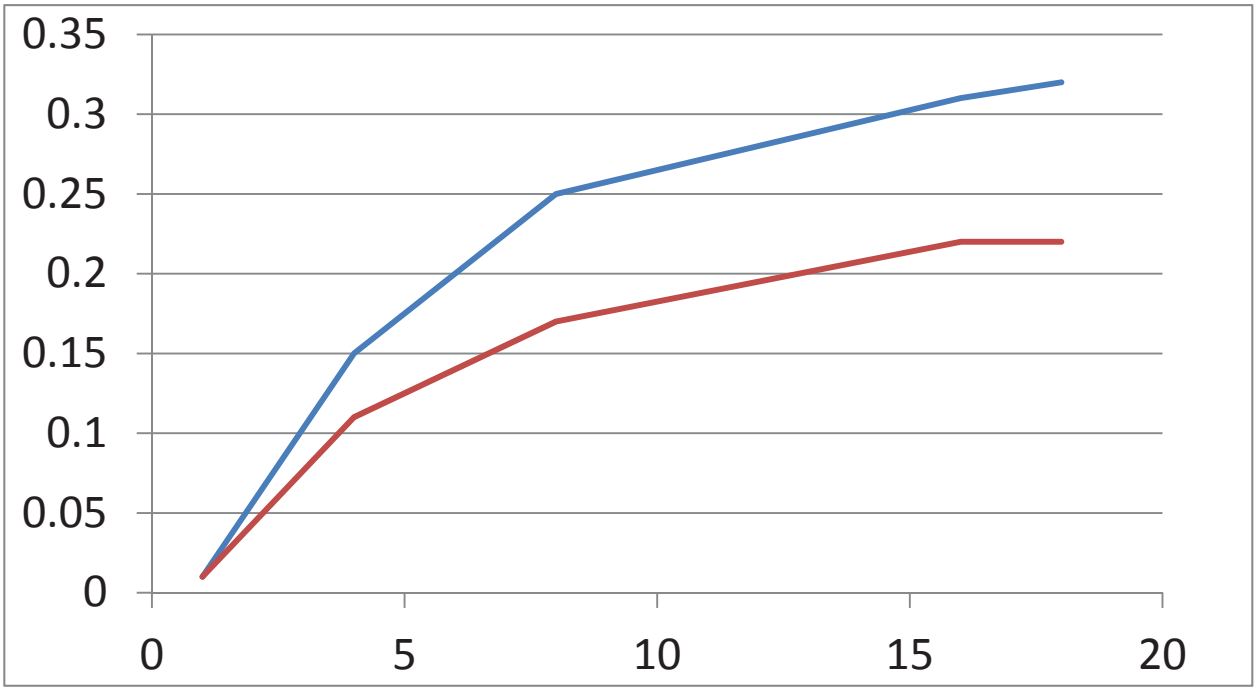
The strains were inoculated in respective medias, which were then grow to different phases in bacterial growth curve 100 µl of each diluted culture was pipeted into each wells in a fresh microtiter. The plates were covered and incubated at optimal growth temperature overnight or 16-18 hrs.

Planktonic bacteria was removed from each microtiter dish by thorough 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 the 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 followed by resuspension in glacial acetic acid

GROWTH CURVE ANALYSIS

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

GROWTH CURVE FOR *Cronobacter Sakazakii*



RED LINE- CULTURE IN SHAKING OR IN PLANKTONIC MODE

BLUE LINE -CULTURE IN NON SHAKING CONDITIONS OR BIOFILM MODE

RESULTS AND DISSCUSSIONS

The red line in graph represents the culture in shaking conditions or planktonic mode whereas blue color represents culture in non shaking condition or biofilm mode. From our graph we concluded that absorbance increases for the culture kept in non shaking (biofilm) mode due to formation of slime or EPS. This culture is clearly visible along the surface of walls if visualised carefully absorbance It also represents that it takes around 16 to 24 hours for the growth of Cronobacter Sakazakii

PROTEOMIC ANALYSIS

MATERIALS-

BACTERIAL STRAINS- *E.coli* being used as a negative control(not known to form a biofilm) and *Cronobacter Sakazakii* (biofilm forming)

MEDIA- Tryptic soy broth for *Cronobacter Sakazakii* and luria broth(LB) for *E.coli*
CHEMICALS- PBS(phosphate buffered saline)(NaCl+KCl+ KH2PO4+NA2HPO4) in about 800 ml of water and making volume upto 1000l at Ph 7.4, sample loading dye(tris Hcl at Ph 6.8+SDS+glycerol+beta mercaptoethanol+ EDTA+bromophenol blue)
SDS gel running chemicals were prepared which includes Tris base at Ph (8.8), Tris base at Ph (6.8),10% ammonium persulphate(APS), Acrylamide, sample buffer(Tris base+ glycerol+ bromophenol blue & SDS in distilled water) , running buffer at Ph (8.3 to 8.7) (tris base+ glycine+ SDS in water) coomasie blue for staining and glacial acetic acid, methanol for destaining.

INSTRUMENTS- water bath,SDS gel running equipments equipments spectrophotometre,pippettes, tips,petriplates etc.

METHOD-

Strains for both *E.coli* and *Cronobacter* were innoculated in their corresponding media that is TSB for *Cronobacter* and LB for *E.coli* for around 48 hrs in petriplates . One set of petriplates were kept in shaking conditions to analyse for planktonic stage and other set was kept simple in non shaking conditions to analyse for biofilm stage at 37 degree celcius in an incubator.

After about 48 hours the petriplates kept at non shaking mode were taken and given a wash with PBS buffer to remove planktonic cells followed by centrifugation , similarly pellets were obtained for culture kept at non shaking conditions were given a wash with PBS buffer. The samples were suspended in SDS loading dye and were kept at boiling water bath at 100 degree celcius for about 5 minutes followed by SDS gel running.

SDS GEL RUNNING

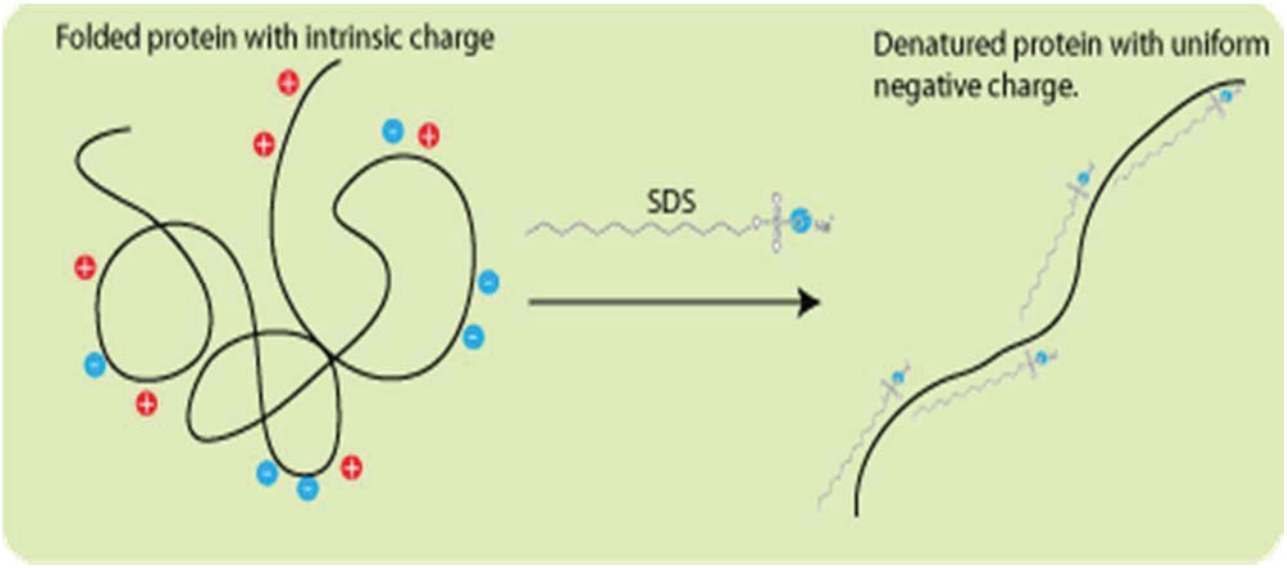
WORKING PRINCIPLE- SDS gel running is generally based on presentation of protein structures. The process of separation of macromolecules in an electric field is called *electrophoresis*. A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The method is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). It was first discovered by U.K Laemmli

SDS (also called lauryl sulfate) is an anionic detergent, meaning that when dissolved its molecules have a net negative charge within a wide range of pH . A polypeptide chain binds amounts of SDS in proportion to its relative molecuar mass. The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode a positively charged electrode in an electric field

SDS is a detergent that is present in the SDS-PAGE sample buffer where, along with a bit of boiling, and a reducing agent (normally DTT or B-ME to break down protein-protein disulphide bonds, it disrupts the tertiary structure of proteins. This brings the folded proteins down to linear molecules.

SDS also coats the protein with a net uniform negative charge, which masks the intrinsic charges on the R-groups. SDS binds fairly uniformly to the linear proteins (around 1.4g SDS/ 1g protein), meaning that the charge of the protein is now approximately proportional to it’s molecular weight.

SDS is also present in the gel to make sure that once the proteins are linearised and their charges masked, they stay that way throughout the run.



THE ROLE OF SDS

(image source:bitsizebio.com)

GEL MATRIX- In an applied electrical field, the SDS-treated proteins will now move toward the positive anode at different rates depending on their molecular weight. These different mobilities will be exaggerated in the high- friction environment of an gel matrix.

As the name suggests, the gel matrix used for SDS-PAGE is polyacrylamide, which is a good choice because it is chemically inert and crucially, can easily be made up at a variety of concentrations to produce different pore sizes giving a variety of separating conditions that can be changed depending on the needs.

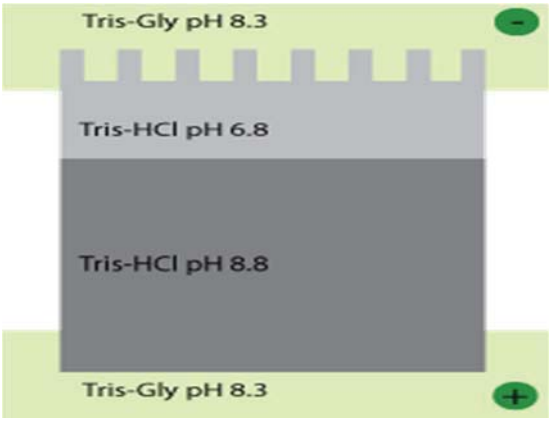
A BUFFER SYSTEM AND A STACKING GEL-To conduct the current through from the cathode (negative) to the anode (positive) through the gel, a buffer is needed. Typically, the system is set up with a stacking gel at pH 6.8, buffered by Tris-HCl, a running gel buffered to pH 8.8 by Tris-HCl and an electrode buffer at pH 8.3. The stacking gel has a low concentration of acrylamide and the running gel a higher concentration capable of retarding the movement of the proteins.

Since glycine can exist in three different charge states, positive, neutral or negative depending on the pH. This is shown in the diagram. Control of the charge state of the glycine by the different buffers is the key to the whole stacking gel thing.

When the power is turned on, the negatively-charged glycine ions around pH 8.3 electrode buffer are forced to enter the stacking gel, where the pH is 6.8. In this environment glycine switches predominantly to the zwitterionic (neutrally charged) state. This loss of charge causes them to move very slowly in the electric field.

The Cl- ions (from Tris-HCl) on the other hand, move much more quickly in the electric field and they form an ion front that migrates ahead of the glycine. The separation of Cl- from the Tris counter-ion (which is now moving towards the anode) creates a narrow zone with a steep voltage gradient that pulls the glycine along behind it, resulting in two narrowly separated fronts of migrating ions, the highly mobile Cl- front, followed by the slower, mostly neutral glycine front.

All of the proteins in the gel sample have an electrophoretic mobility that is intermediate between the extreme of the mobility of the glycine and Cl- so when the two fronts sweep through the sample well the proteins are concentrated into the narrow zone between the Cl- and glycine fronts.



A BUFFER SYSTEM

(image source:bitsizebio.com)

This procession carries on until it hits the running gel, where the pH switches to 8.8. At this pH the glycine molecules are mostly negatively charged and can migrate much faster than the proteins. So the glycine front accelerates past the proteins, leaving them in the dust. The result is that the proteins are dumped in a very narrow band at the interface of the stacking and running gels and since the running gel has an increased acrylamide concentration, which slows the the movement of the proteins according to their size, the separation begins hence the proteins in sample would all enter the running gel at different times, resulting in very smeared bands.

So the stacking gel ensures that all of the proteins arrive at the running gel at the same time so proteins of the same molecular weight will migrate as tight bands. Once the proteins are in the running gel, they are separated because higher molecular weight proteins move more slowly through the porous acrylamide gel than lower molecular weight proteins. The size of the pores in the gel can be altered depending on the size of the proteins we want to separate by changing the acrylamide concentration.

Polyacrylamide gels tend to restrain larger molecules from migrating as fast as smaller molecules Since the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides.

Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions. The purity of protein samples can be assessed and the progress of fractionation or purification procedure can be followed. Different staining methods can be used to detect rare proteins and to learn something about their biochemical properties. Specialized techniques like Western blotting, two-dimensional electrophoresis, and peptide mapping can be used to detect extremely scarce gene products, to find similarities among them, and to detect and separate isoenzymes of proteins.

STAINING AND DESTAINING

STAINING-

The gel obtained was stained in coomasie blue solution

PRINCIPLE-

The Coomassie dye bind to proteins through ionic interactions between dye sulfonic acid groups and positive protein amine groups as well as through Van der Waals attractions. The capability coomasie is due to its particular properties.It has a unique property it manifests a leuco form below pH 2. Solutions of the dye, dark blue black at pH7 turn a clear tan upon acidification.

The leuco form recovers its blue color upon binding to protein, apparently due to the more neutral pH of the environment around the protein molecule. Under proper conditions, a gel placed in an acidified solution of Coomassie will manifest blue protein bands on a light amber background. The bands develop rapidly with the background color is so light as to be essentially clear.

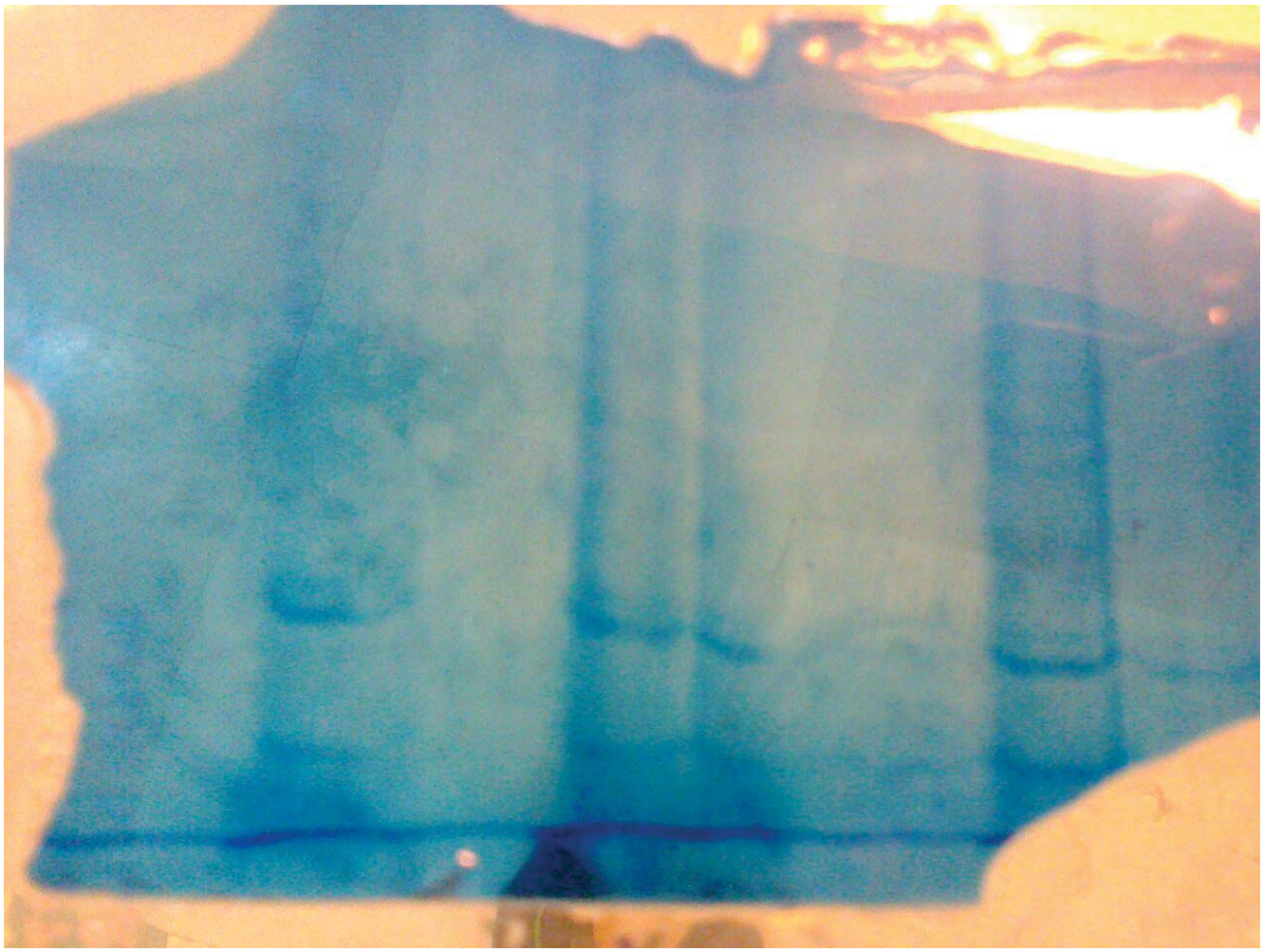
Staining is complete when the gel is no longer visible in the dye solution. Prior to complete staining, the gel will appear as a lighter area against the dark staining solution.

DESTAINING-

After keeping the SDS gel for around about 24 hours in a staining solution of coomasie blue until dark bands are quite visible after 24 hours the gel is kept in a destaining solution consisting of methanol and glacial acetic acid mixed in distilled water(400ml of methanol+100 ml of glacial acetic acid in 500ml of DH_2O)

The bands were obtained as shown in picture as-

RESULTS & DISSCUSSIONS



The proteins band were observed as shown. The bands were discovered and the bands that we obtained were similar in all biofilm and planktonic mode and same for both E.coli and Cronobacter samples

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 proteomic analysis had been useful in knowing the protein profile expressions of different modes. The proteins involved in biofilm formation and responsible for different types of virulent factors is a key and important factor in determining the proteins that are responsible for virulence of pathogenic biofilms.

Although yet not complete information is yet available regarding the factors that are responsible for and are involved in formation of biofilm and its virulence hence it thereforse forms a major research target in field of modern era for genomics and proteomics.

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