Pseudomonas aeruginosa gene expression

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Pseudomonas aeruginosa is the gram negative rod shaped bacteria is found in all natural and man made environments. the organism is so stubburn so that it can grow in any environment and also at high temperatures such as 420C. The unique ability of the organism is forming biofilm. The biofilms are formed by the organism with the association with a surface. The surface may be anything such as cloth, paper, glass etc and any artificial surface. It is the opportunistic pathogen which behaves neutral to the normal tissues but it infects once the defences of them are compromised. The development of the biofilm starts with the attachment of the freely moving planktonic cell to a surface. Generally the twitching motility of the P. aeruginosa helps to use its unipolar flagellum to adhere a surface. The bacterium develops colonies known as microcolonies and adhere to the surface strongly which are known as pili structures (Baron S et al., 1996). During the biofilm development several factors plays a key role mainly the exopolymeric matrix. The EPS matrix is not visualised directly, but it can be done by using some fluorescent dyes. The functioning of the EPS matrix in holding cells of Pseudomonas aeruginosa together and forming the mature biofilms. The biofilm develops with cell division and recruitment. The cells interacts via quorum sensing and these interactions are studied under scanning electron microscopy. The biofilm formation takes place in stages and many changes are observed in its size and shape during the development. The EPS matrix is made up of polysaccharides, proteins and nucleic acids. The exopolysaccharide matrix is the important component of the biofilm. The exopolysaccharide genes such as pilB, pslA, lecA, ureB and alginate genes are responsible for the formation of pseudomonas biofilms. The locus of the

psl polysacchide genes encode the cells to adhere to a surface and thus maintaining the biofilm structure.

In this research project we try to quantify the gene expression of the above genes pilB, pslA, lecA, ureB using rpoD as the house keeping gene. The biofilms are grow invitro on microcarriers. The microcarriers made up of dextrons in the range of 60-90µm in size are used as a surface for Pseudomonas aeruginosa to form biofilms. The microcarriers are spherical in size where the bacteria can easily adhere on surface. In this present work we try to grow planktonic cultures and biofilms cultures on the microcarriers in a fermentor separately. The genes responsible for the biofilm formation are quantified by comparing the planktonic and biofilm samples. The planktonic cells grow very rapidly whereas the bacterial adhering takes some time to form biofilm in other case. The samples are collected at regular intervals and mRNA is extracted. The gene expression is quantified using real time PCR. The relative quantification method of qPCR or real time PCR method does not require any standard curve for the analysis where the samples are normalised by using an internal control. The threshold CT values obtained from the curve are used to calculate relative quantification by comparative âˆ†âˆ†CT method. The results from the planktonic and biofilm gene expression results are compared. On the other hand we also assay the nutrients such as glucose, ammonia, phosphorus and protease during the fermentation. The concentration levels of the nutrients are also compared for both biofilm and planktonic fermentation.

Review of literature:

Pseudomonas aeruginosa is a member of gamma proteobacteria class of bacteria. The bacteria is gram negative, rod shaped and aerobic belongs to the bacterial family of pseudomonadanceae. The recent developments in the taxonomy based on the 16s ribosomal RNA or conserved macromolecules, the family members are divided into eight groups. Pseudomonas aeruginosa is a species present in this group sharing the with other twelve members.

Pseudomonas aeruginosa is a free-living bacterium, commonly found in soil, water, skin flora and in all man made environments. The organism regularly occurs on the surfaces of the plants and animals. Pseudomonas aeruginosa is an occasional pathogen to plants. It is also an increasingly emerging pathogen of clinical relevance. The organism not thrives in the normal atmosphere but also in the places with little oxygen and thus forms the colonies in natural and artificial environments.

Pseudomonas aeruginosa is an opportunistic pathogen for humans, which means that it exploits some break in the host defences to initiate an infection. The bacteria never infects the uncompromised tissues, but it can infect any if the tissue defences are comprised. It can cause infections such as urinary tract, respiratory system infection, soft tissue infection dermatitis, gastro intestinal infections. It causes a variety of systemic infections in patients particularly with severe burns and immune suppressed in cases of cancer, AIDS, cystic fibrosis in lungs.

Characteristics

Pseudomonas aeruginosa is rod shaped gram negative bacteria. The size of it ranges between 0. 5 to 0. 8 µm by 1. 5 to 3. 0 µm. almost all the strains of Pseudomonas are motile with a unipolar flagellum. It is ubiquitous in soil , water and on the surfaces in contact with water or soil. It has respiratory metabolism and can grow in the absence of oxygen if NO3 is available as respiratory acceptor for electron.

Generally the bacterium is found in nature in the form of biofilm attached to a surface or substrate, or in the planktonic form as a unicellular organism swimming actively with its unipolar flagellum. It is one of the most vigorous and fast swimming organism found in pond waters. It can grow at temperatures at 42OC or high, but the optimum temperature is 30 to 37OC. it is resistible to a wide variety of physical conditions such as temperature and can even resist the salts, dyes, antiseptics and most commonly used antibiotics.

Pseudomonas aeruginosa produces different types of colonies from the source they have isolated. They appear like small and rough colonies. Moreover the samples collected from the clinical sources look like a fried egg like structure which is smooth, large with elevated appearance. The other type of colonies obtained from the respiratory and urinary tract secretions looks like mucoid. this mucoid appearance is attributes for the production of alginate slime. These smooth, mucous colonies presumably plays an important part in the colonization and virulence.

Pseudomonas aeruginosa produces two types of soluble pigments. Pyocyanin which is a blue-green and a yellow-green and fluorescent pigment called as pyoverdin. Pyocyanin is produced abundantly in the media with low levels of iron and also functions for the iron metabolism of the organism. Many strains of Pseudomonas aeruginosa produces pyocyanin but not all the strains does it. Although the organism is classified as the aerobic, sometimes the organism is considered as facultative anaerobe where it can adapt and proliferate the conditions with less or total O2 depletion. The organism can grow in the absence of oxygen if NO3 is available as respiratory acceptor for electron. It can ferment arginine by substrate level phosphorylation where the layers of alginate present surrounding the mucoid cells of the bacteria controls the diffusion of oxygen(Collins FM, 1955), this is witnessed in the cystic fibrosis of the lung infection. Pseudomonas aeruginosa which is found in the biofilm form is responsible for a variety of activities involved which are natural and artificial.

Resistance to antibiotics

Pseudomonas aeruginosa is opportunistic pathogen, has a notorious resistance to antibiotics. The organism is very resistance towards many antibiotics due to the potential permeability barrier provided by the gram negative membrane. Pseudomonas aeruginosa in the biofilm form makes the cells impervious to the concentrations of the therapeutic antibiotic. The organism gets associated with others like actinomycytes, moulds and bacillus because of the natural environment they live. Moreover the organism have antibiotic resistance plasmids R factors and RTF’s, the genes

are able transferred by conjugation and transduction following the mechanism horizontal gene transfer(HGT) (Todar. K, 2008).

Biofilms

Biofilms are defined as a matrix enclosed bacterial populations which are adherent to each surfaces or interfaces. Microbial aggregates and floccules and also adherent populations within the pore spaces of the media are spoken in the definition (Costerton J. W, et al 1995). bacterial biofilms came into existence and became significant from their first recognition in their ubiquity. The past researches show that profoundly the growth phase of the biofilms is distinct from the planktonic. In response to the surfaces proximity, bacterial cells alter their phenotypes (Costerton J. W, et al, 1995). The early stages of biofilm formation the bacteria is in stable juxta position with those of same and other species. The micro environmental conditions, cellular juxtapositions and the extra cellular polysaccharide matrix constitute to the development of the bacterial biofilm. Different bacteria responds to the specific environmental conditions with certain growth patterns, and a structurally complex and mature biofilm develops(Hamilton W. A et al, 1987). Physiological cooperation is the major criteria in shaping structure and juxta positions establishment to make biofilms adherent for the surfaces. It has been proposed that large suites of genes are expressed differentially regulated.

Formation

The formation of the biofilm initially starts with the attachment of the freely floating bacterial cells to a surface. The bacterial colonies adhere to the surface with weak vander wall forces. These colonies can lead themselves to

more adhesion called as pili structures (Baron S et al., 1996). The organism adheres more to the available surface by binary fission, exopolymeric matrix is produced to develop mature biofilms. The matrix produced holds the biofilm together. Biofilm develops with cell division and recruitment, the cell in the biofilm communicate via quorum sensing. We can observe changes in shape and size in the final stages of biofilm formation.

Distribution and ubiquity of biofilms

The general phenotypic strategy of organism is to change in the mode of growth that to adopt to the altering growth conditions. The cells of Pseudomonas aeruginosa which is a ubiquitous bacterial species respond to all favourable nutrient conditions. The organism adheres to the available surface by binary fission, exopolymeric matrix is produced to develop mature biofilms. The rod shaped bacteria grows in matrix which is enclosed in a sessile growth mode. The cells are protected from the biological, antibacterial, chemical agents and adverse environmental conditions. The periodically shed planktonic cells from the biofilm provide and sacrifice the protection to the new fresh habitats can colonize with the biofilm. The adherent biofilm populations has a significant metabolic activities(Fletcher. M, 1986).

The extent of biofilm formation is predicted in the particular system is based on the following principles:

- Metabolically active bacteria show tendency towards the surfaces for adhesion in all natural environments.

- In all environmental conditions the extent of biofilm formation is controlled by the available nutrient for cell respiration and exopolysaccharide production.
- The organic nutrients sometimes tend to associate with the surfaces, in such cases the local biofilm is difficult to trigger. This is more in extremely oligotrophical environments, but bacteria normally does not adhere to surfaces in nutrient less environments.

Pseudomonas aeruginosa Biofilms

The communities of microbial bacteria(Pseudomonas aeruginosa) forms a definite structures with the association with surfaces are abundantly available environment. These structures are often called as biofilms which are less susceptible to antimicrobial agents. These biofilms causes chronic infections and very difficult to remove. The extracellular polymeric matrix(EPS) are known to holds the biofilm structure and it performs as a matrix. The EPS matrix holds the biofilm cells together and protects them from shear forces in fludic environments. The extra cellular matrix for Pseudomonas aeruginosa forms the biofilm development in different stages. The matrix holds the cells together on the surface and forms a critical structural design resists to many environmental changes. Pseudomonas aeruginosa biofilm develops in a five stage multicellular cycles, the process is initiated by the attachment of planktonic (free) cells to a surface. This is followed by the formation of microcolonies. The microcolonies are seeded and gets dispersed where the cells form the microcolonies comes out to occupy a new surface(Ma. L et al, 2009; Stoodley. P et al, 2002).

The EPS of Pseudomonas aeruginosa composed of polysaccharides, proteins and nucleic acids. It is the important component of the biofilms. The exo polysaccharide genes lec A , Pil B, Psl A, alginate are responsible for the formation of Pseudomonas biofilms. The locus of the Psl exopolysaccharide encodes bacterial cells to surface and thus maintaining the biofilm structure. The matrix which is anchored by the Psl is not visualised directly during the developmental stages of Pseudomonas aeruginosa biofilm.

In addition to the present scenario of exopolysaccharides, extracellular DNA also plays an important part in . P. aeruginosa biofilm matrix (Allensen-Holm M et al, 2006). The extracellular DNA is derived from the chromosomes and it functions for signalling between the cells interconnecting the biofilm matrix. In the biofilm matrix, extracellular cellular DNA promotes cation gradients, release of genomic DNA and induces antibiotic resistance (Mulcahy H, et al. 2008).

Real time PCR

Polmerase chain reaction or simply PCR is introduced by Kary mulis in 1980, which really a revolution in the molecular biology. PCR is the standard procedure to amplify small quantities of DNA molecule into large quantities. The method depends on thermal cycling which involves the continuous heating and cooling of reaction for DNA melting and enzymatic replication of DNA. PCR applications employ a general heat stable DNA polymerase such as Taq polymerase enzyme isolated from the bacterium Thermus aquaticus.

PCR method is involves three major steps. 1) Denaturation 2) Annealing 3) Elongation. In the first step DNA template is denaturated to single strand at

high temperature of 940C. In the second step, single stranded DNA template annealed with primers, the temperature is 650C. At 720 C the DNA starts elongation which is third step. DNA polymerase synthesizes new complementary strand to the template DNA strand.

Real time polymerase chain reaction or qualitative polymerase chain reaction is a laboratory technique which not only amplifies but also quantifies the target DNA molecule. it helps the user for detection and quantification(absolute and relative) of many specific genes in a present in a given DNA sample. The general principle of polymerase chain reaction (PCR) is also used in the RT PCR. Unlike PCR, RT PCR is a new technology product is detected at the end of the reaction.

There are two common methods for the product detection in RT PCR

1) Non -specific fluorescent dyes which interacts with the double stranded DNA molecule

2) Sequence specific DNA probes consists of oligonucleotides that are fluorescently labelled with reporter. The product is detected after the hybridization of the probe and complementary strand.

The RT PCR is used to amplify the reversly transcribed cDNA products from the mRNA, which is becoming the effective and powerful tool in the field of molecular biology to quantify and study the gene expression. The RT PCR method is very easy to apply provides the reliable, accuracy and rapid gene expression quantification. The accurate nucleic acid quantification requires mathematical application for data analysis(Michael W. P., 2001). The real

time PCR provides the very accurate and reproducible quantification of gene copies (Heid C. A., 1996). Unlike other PCR methods, RT PCR does not require post process sample handling, prevents product contamination, provides faster and accurate assay results. Real time PCR is very accurate and less labour usage than the present gene quantification methods. Nucleic acid sequence quantification has an important role in the biological research. Measuring the gene expression have been extensively used in the biological responses to various external stimuli(Tan et al, 1994; Huang et al, 1995). Gene and genome quatification also used for HIV demonstrates the change in the expression level of virus in the disease period(Conner et al. 1993); (piatak et al, 1993).

There are two types of quantification methods used in real time PCR:

- Absolute quantification
- Relative quantification

Absolute quantification determines the input copy number generally by relating the PCR signal to a standard curve. In this method equal input amounts of sample, each sample under analysis amplifies with identical efficiency up to the point of qualitative analysis(Heid et al, 1996). We quantitate the unknowns based on a known standard quantity. The unknown samples are compared to the known by creating a standard curve. The input copy number of the PCR signal is related to standard curve in absolute quantification method. Sometimes it is not necessary to know the copy number but it is important to calculate the relative change gene expression (Livak. K. J et al, 2001).

The second method is relative quantification, we analyse the changes in gene expression in a given sample relative to another reference sample(untreated control sample). Relative standard again consists of two types of methods they are relative standard curve and comparative Ct method. In the relative quantification the PCR signal is related to the targeted transcript of the samples in the group. The new applications of comparative Ct method is the 2-âˆ†âˆ†Ct method which is efficiently useful for the analysis of qPCR data.

The 2-âˆ†âˆ†Ct method(Livak. K. J et al , 2001):

Derivation of 2-âˆ†âˆ†Ct method:

The equation for the amplification of PCR can be written as:

Xn= X0 (1+Ex)n————————————————————————(1)

Where Xn is the number of target molecules at cycle n of the reaction.

X0 is the initial number opf target molecules.

Ex is the efficiency of the target amplicon.

n is the number of cycles

CT is the threshold cycle, indicates the fractional cycle at which the amount of amplified target reaches a fixed threshold.

XT = X0 (1+Ex)CT. X= Kx————————————————————–(2)

Where XT is the threshold number of target molecules.

CTX is the threshold cycle for target molecule and Kx is constant.

A similar reaction for the internal control gene is

RT = R0 (1+ER)CTR= KR————————————————————-(3)

Where RT is the threshold number of reference molecules

R0 is the initial number of reference molecules

ER is the efficiency of the reference molecule.

CT. R is the threshold cycle for reference amplicon.

KR is a constant.

Dividing XT and RT, we get ,

(XT/ RT)= (X0 (1+Ex)CT. X) / (R0 (1+ER)CT. R)= (Kx / KR) = K

————————-(4)

For real time amplification using Taqman probes the exact values of the XT and RT depend upon the number of factors including the reporter dye and fluorescence properties of the probes, efficiency and purity of the probe, florescence threshold setting. Thus k cannot be equal to one. Assume the efficiencies of target and the reference as equal.

ER = Ex= E

(X0 / R0) (1+ E) CT. X- CT. R = K————————————————(5)

XN (1+E)-âˆ†CT = K————————————————————-(6)

Where XN is equal to the normalised amount of target gene (X0 / R0) and âˆ†CT is the difference of the threshold cycles of target and reference (CT. X-CT. R).

The expression can be written as

XN= K(1+E) -âˆ†CT ————————————————————-(7)

If we divide the whole equation by XN for any sample q by XN as the calibrator(cb)

XN. q /XN. cb ={[K(1+E) -âˆ†CTq]/[K(1+E) -âˆ†CTcb]}

=(1+E) -âˆ†âˆ†CT

Here -âˆ†âˆ†CT = -(âˆ†CTq -âˆ†CT. cb)

For amplicons which are designed less than 150 bp, primer and magnesium concentration are optimised, the efficiency is nearly equal to 1. Therefore the amount of target gene, normalized to an endogenous reference which is relative to a calibrator, taken as

Amount of target = 2-âˆ†âˆ†CT .

When the threshold is set for n standard deviations in the plot , above the mean of the base line emission from cycles 0 to n. The standard deviations is calculated from the data attained from base line of the amplification plot. When the fluorescence exceeds the threshold limit, CT values are determined at that point. The CT values are ten times more than the

standard deviation of base line. The point at which amplification plot crosses the threshold can be defined as CT (Heid et al, 1996).

Materials and methods

Identifying pseudomonas aeruginosa

Gram straining:

Prepare a small amount of smear on a clear glass slide.

Heat fix the smear to slide.

Add a drop of crystal violet on to smear allow for 60 seconds.

Rinse the slide with water.

Add a drop of grams iodine to the smear allow for 60 seconds.

Rinse the slide with water.

Add 95% absolute ethanol to destain the crystal violet. Rinse again with water.

Add a drop of saffronin over the smear, allow for 60 seconds. Rinse with water.

Blot dry the slide, do not rub. Observe the smear under microscope.

10. If the colour of the bacteria appears pink it is gram negative, it is gram positive if it appears purple. Rod shaped pink coloured bacteria is clearly observed for gram negative pseudomonas aeruginosa.

Shake flask method

Prepare media of nutrient broth about 13 grams per litre of distilled water. Mix well and pour 50 ml of media into a 250ml conical flask. Plug the conical flask and wrap the mouth with aluminium foil. The less volumes of media in the flask increases surface area and oxygen transfer rate(OTR). The flasks are autoclaved at 1210 C and 1. 5 lbs pressure for 1 hour 15 minutes.

Planktonic-contains 50 ml of nutrient broth

Biofilm-contains 25ml of PBS with microcarriers plus 25 ml of nutrient broth.

Inoculate a loopful of pseudomonas aeruginosa into 50 ml nutrient broth aseptically. Allow the bacteria to grow overnight. Take the optical density at 550nm using spectrophotometer. The amount of the inoculums to be added to the shake flasks for analysis is calculated using the formula below:

Amount of sample to add into shake flask

= [working concentration X volume(ml)] / stock(OD) obtained

After inoculation the flasks are kept in the orbital shaker at 300C. The samples of 1 ml is collected for RNA extraction at particular time intervals at 0, 1, 2, 4, 24 and 48 hours for planktonic. In respect the biofilm samples are collected at time 0, 22, 24, 26, 44, 46 and 48 hours as their growth is slower than the freely suspended planktonic cells. Then 2 ml of RNA later solution is added to the collected samples for RNA stabilization. The optical density is regularly monitored to check the growth of pseudomonas aeruginosa .

Fermentation

The fermentation process we perform here is batch fermentation. The bioreactor used for the study is a small scale fermentor from APPLICON BIOTECHNOLOGIES. The process will take place in a 3L fermentor(consists the working volume of 2. 5L) for about 24 hours. During the run the criteria of temperature is kept at 300C . The pH is run free, stirrer speed is set at 600rpm for planktonic culture. The fermentor is run free for 24 hours overnight to check the production of foam. The cold finger helps to control the temperature by feeding water from/to the reactor. The o ring is checked for any leakage. The sample port is a tube for sampling using back pressure and forces the liquid into a glass recipient. The inlets for acid, alkali and antifoam are linked to durham bottles, going through the pumps. The durham bottles are blank, because above parameters are not controlled in this process. Air is pumped through a filter. The condenser is connected to water that comes out of the fermentor. When the water is warmer than outside, condensed water reaches the condenser. The gas goes from an outlet connected to the condenser.

The dissolved oxygen probe is connected to dO2 cable from the machine. It measures the amount of dO2 in the media. This probe needs to be kept in the buffer, when not in use. The probe should be plugged for 10 to 12 hours before the inoculation inorder to polarize. The stirrer is placed on the top of fermentor. The vessel is heated with the help of heating pad and temperature is detected using temperature probe. The air inlet is set up through the pumps and its volume is controlled with a rotometer in front of the machine. In this case 2. 5L of air/min is supplied to the fermentor. For the

gas analysis of oxygen and carbon dioxide the gas is passed through a drying column and reaches the monitor which is already set up for calibration. we run two runs of fermentor for both planktonic and biofilm cultures. The parameters such as temperature, aeration for planktonic and biofilm cultures are almost same. The only parameter varies for both is stirring is maintained at 600rpm for planktonic and 200rpm for biofilm fermentation.

Procedure:

The fermentor is sterilised and all the valves are sealed with foil before going into the autoclave. The fermentor is run for about 24 hours before adding the inoculum to check any formation of foam. About 20 ml of an overnight culture is added to the fermentor.

Sampling : About 7ml of sample is collected for every 20min for planktonic and every 2 hours for biofilm until the exponential phase is reached. The sample collected is preserved in the freezer for different analysis. The sample is assayed for gene expression by adding 2 ml of RNA later solution for RNA stability. The samples are assayed for ammonia, phosphorus, glucose and protease. The fermentation conditions are listed below.

RNA EXTRACTION (protocol provided by applied biosystems)

Materials required:

2-mercapto ethanol, 100% ethanol, 10% SDS(in RNase free water), 0. 5 µl/sample.

Lysozyme solution:

100 µl/ sample

10mM Tris HCl (PH 8. 0)mM EDTA

10mg/ml lysozyme (in RNase free water)

Lysis buffer:

Before beginning the lysis and homogenization steps, prepare a fresh amount of lysis buffer containing 1% 2-mercapto ethanol for each purification procedure. Add 10µl 2-mercaptoethanol for each 1ml lysis buffer. Use 350 µl of freshly prepared lysis buffer for 1×109 bacterial cells.

Lysis and homogenization:

Harvest 1×109 bacterial cells and transfer them to an appropriately sized microcentrifuge tubes. Centrifuge at 500xg for 5 minutes at 40C to pellet cells. Discard the supernatant. Add 100 µl of prepared lysozyme solution to the cell pellet and resuspend by vortexing. Add 0. 5 µl 10% SDS solution vortex to mix well. Incubate the cells in the tube for 5 minutes at room temperature. Add 350 µl lysis buffer prepared with 2-mercaptoethanol. Vortex to mix well. Transfer the lysate to a homogenizer inserted in an RNase free tube and centrifuge at 12, 000xg for 2 minutes at room temperature. Remove the homogenizer when done.

Purifying the RNA from bacterial cells:

These steps are followed to bind wash and elute the RNA from the P. aeruginosa cells sample.

Add 250 µl 100% ethanol to each volume of bacterial cell homogenate.

Mix thoroughly by vortexing to dispersing any visible precipitate that may form after adding ethanol.

Transfer the sample(including any remaining precipitate) to a spin cartridge(with a collection tube.

Centrifuge both the spin cartridge and collection tube at 12, 000xg for 15 seconds at room temperature. Discard the flow through and re-insert the spin cartridge in the same collection tube.

Add 700 µl of wash buffer I to the spin cartridge. Centrifuge at 12, 000xg for 15 seconds at room temperature. Discard the flow through and the collection tube. Place the spin cartridge into a new collection tube.

Add 500 µl wash buffer II with ethanol to the spin cartridge.

Centrifuge at 12, 000Xg for 15 seconds at room temperature. Discard the flow through and re insert the spin cartridge into the same collection tube. The steps are repeated once again.

Centrifuge the spin cartridge and collection tube at 12, 000Xg for 1 minute at room temperature to dry the membrane with attached RNA. Discard the collection tube and insert the spin cartridge into a recovery tube.

Add 50 µl of RNase free water to the centre of spin cartridge.

Incubate at room temperature for 1 minute.

Centrifuge the spin cartridge and recovery tube for 2 minutes at 12, 000Xg at room temperature.

Store the purified RNA or proceeded for DNase treatment after RNA treatment.

(store purified RNA at -800C for long term).

Agarose gel electrophoresis:

The RNA samples after extraction are checked for the RNA signal. To visualise the RNA 1% agarose gel electrophoresis. The materials required for agarose gel electrophoresis is TAE buffer and agarose.

TAE buffer(50x Tris acetate)

242 g of Tris base

57. 1 ml glaciel acetic acid

100 ml of EDTA 0. 5M PH . 8. 0

700ml of dH2O .

About 20 ml of above solution is mixed with 980ml of distilled water to make it 1X. 1 gram of agarose is added to 100 ml of TAE buffer. The solution is mixed well. The solution is heated until the agarose is completely dissolved and solution becomes colourless. About 2. 5 µl of ethidium bromide is added to the solution to visualise the RNA bands. Special care is taken while adding the ethidium bromide because it is bio-hazardous. Then about 5 µl RNA sample is mixed with 1 µl of DNA loading dye. The RNA samples are RUN with the potential difference of 80V with DNA Hyper ladder II as a standard.

DNase treatment (protocol and kit provided by applied biosystem)

The DNase I treatment is performed to remove any traces of genomic DNA from the eluted RNA.

Protocol:

Add 10x DNase buffer and DNase I to the RNA sample in the following composition:

Amount component

1/9 th volume 10x DNase buffer

4 µl DNase I

The components are mixed with gentle pippeting. Then the mixture is incubated at 370C for 90 minutes. DNase inactivation reagent is added to the solution and mixed well, leave at room temperature for 2 minutes. The samples are centrifuged at maximum speed for 1 minute to pellet the DNase inactivation reagent, after that the RNA solution is transferred to a new RNase free tube. The samples are run on 1% agarose gel electrophoresis to check the DNA contamination. We fou