In vitro Evaluation of the Scope of an Indigenously Produced Polyhydroxyalkanoate Based Co-Polymer, Produced From *Pseudomonas MNNG- S*, in Cell Culture and Virology

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

SHABNA. A

2010PHXF100H

Under the Supervision of

PROF. H. N.MADHAVAN

&

Under the Co-supervision of

PROF. SUMAN KAPUR

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

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CERTIFICATE

This is to certify that the thesis entitled **"In vitro Evaluation of The Scope of an Indegenously produced polyhydroxy alkanoate based copolymer,Produced from Pseudomonas MNNG-S, in cell culture and virology"** submitted by **MS. Shabna.A** ID No **2010PHXF100H** for award of Ph. D. Degree of the Institute embodies original work done by her under our supervision.

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Recite in the name of your Lord who created-
Created man from a clinging substance.
Recite, and your lord is the most generous-
Who taught by the pen-
Taught man that he knew not.
No! (But) indeed, man transgresses
Because he sees himself self-sufficient.
Indeed, to your Lord is the return
(The first revelation to Prophet Mohammad (PBUH) from the Lord)

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Shabna.A

ABSTRACT

The main focus of the present study was to evaluate the scope of novel polyhydroxyalkanoate (Polyhydroxy propionate co- poly hydroxy ocatadecanoate co-polymer) based scaffold derived from a mutant strain of *Pseudomonas* sp. MNNG-S in supporting continuous cultures and primary corneal limbal stem cell culture. The study also validated the potential of the cell culture system, developed with this novel polymer, in supporting viral (HSV-1) cultivation in comparison with other commercially available 3d polymer scaffold systems namely matrigel, mebiolgel and hydro matrix peptide hydrogel.

The polymer was produced from mutant form of the indigenous species of *Pseudomonas* sp. LDC-5 named *Pseudomonas* sp.MNNG-S using a 14l scale fermenter. The SCL-MCL copolymer Polyhydroxyalkanoate, recovered from *Pseudomonas* sp. MNNG-S was solvent casted in pure form as well as in blends with other biodegradable polymers. The blending experiments were done in order to enhance the optical and processing properties of the polymer to make it suitable for cell culture. Protein adsorption study was done to evaluate the capability of scaffolds for cellular interaction. It is noted that, PHA: PEG blended scaffold showed better adsorption than others. MTT assay, performed with the continuous cultures HeLa, HEp-2, Vero, and McCoy on the polymer blends, supported the above finding. Among the blends PHA: PEG at 60:40 was most favored, as it was culture friendly with a superior rate of cellular viability and hence selected for further studies. Cell proliferation assay with colorimetric BrdU ELISA kit showed increase in cell proliferation over the matrix PHA: PEG than that of control. There were no observable morphological changes of continuous cells grown over matrix PHA: PEG when observed by phase contrast microscopy. HEp-2 cells were enclosed within the matrix when analyzed by SEM. The study thus stated that the scaffold prepared by using the indigenous PHA in combination with PEG supports cell growth better than the conventional plastic surface.

Corneal limbal explants on the polymer gave outgrowth within 24 hours when supplemented with DMEM/F12 with 10% FBS and without growth factors. Differentiated corneal epithelial cells formed a stratified morphology with 3 layers. Early cultures expressed putative limbal stem cell markers, ABCG-2 and Np-63 and cultures at a later stage expressed differentiated corneal epithelial markers *K3*, *K12* and connexin. The decrease in expression of putative stem cell markers stands as a limitation of the study. Pore size (40 μm), obtained by solvent casting, can be brought down to nano dimensions by electrospinning method

which might give a better corneal limbal cell proliferation and longer stemness by providing a more hypoxic environment.

The comparative studies were done, with other commercially available polymers like Matrigel, Mebiolgel, Hydromatrix peptide hydrogel, in evaluating the morphology and rate of proliferation of cells. The commercially available polymers formed spherules of HCE cells whereas the novel PHA based copolymer formed layers of cells. The rate of cellular proliferation on the commercially available scaffolds were comparable to the novel polymer, all showed a superior rate of proliferation when compared to the control 2d culture.

Viral infectivity studies using HSV-1 with HCE cells grown in different systems, Matrigel, mebiolgel and hydromatrix peptide hydrogel and PHA: PEG was performed. PHA: PEG showed a significantly good cellular proliferation with viral multiplication. PHA: PEG system would therefore be appropriate for viral multiplication which would be enhanced by the lower level of expression of the four different TLRs studied. However, Hydromatrix peptide cell culture system seemed to be efficient in eliciting cellular immune response on HSV-1 infection, in terms of TLRs studied, and would be appropriate for the studies on host viral interaction.

To conclude, PHA based co polymer from indigenous species of *Pseudomonas MNNG-S* has a wide future in *in vitro* and *in vivo* applications of cell culture, as it gives, superior viability and proliferation of continuous cultures, differentiating and stratifying capacity of primary corneal limbal stem cell culture and minimal innate immune response elicited in response to infection, in terms of TLRs studied.

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LIST OF ABBREVIATIONS

PGA: Polyglycolic acid	HSV: Herpes Simplex Virus -1
PLA: Poly lactic acid	2d: Two Dimensional
PLGA: Poly lactic-co-glycolic acid	3d: Three Dimensional
PCL: Polycaprolactone	GC: Gas chromatography
PHA: Polyhydroxyalkanoates	MS: Mass spectroscopy
PHB: Poly (3-hydroxbutyrate)	LDC: Lady Doak College
SCL: Short Chain Length (consisting of C3 to C5)	PCR: Polymerase Chain Reaction
MCL: Medium Chain Length	VNTR: Variable Number Tandem Repeats
Multicellular spheroid (MCS)	SNP : Single Nucleotide Polymorphism
LCL: Long Chain Length	DNA: Deoxy Ribonuclic Acid
RWVs: Rotating Wall vessels	HEp-2: Human Epithelial Carcinoma cell line
HIV: Human Immunodeficiency Virus	HCE: Human corneal epithelial cell
FBI CODIS: Federal Bank Investigation	ELISA: Enzyme Linked Immunosorbant assay
RNA: Ribonueclic Acid	TLR: Toll Like Receptor
BSA: Bovine serum albumin	DHPLC : Denaturing High performance liquid chromatography
PAGE : Poly Acryl amide Gel Electrophoresis	NIH: National Institute of Health
NCBI: The National Cell Bank of Iran	BrdU: Bromodeoxy Uridine

TEAA:Tetra Ethyl Ammonium Acetate	WHO: World Health organization
HPV: Human Papilloma Virus	HCV: Hepatitis C Virus
EtO: Ethylene Oxide	UV : Ultra violet
dNTP: deoxy Nucleotide Phosphate	ATCC: American Type Culture Collection
MNNG: N-methyl-N'-nitro-N-nitrosoguanidine	*tk/gcv: Thymidine kinase/gancyclovir*
MMT: Montmorillonite	PHEMA: Poly(2-hydroxyethyl methacrylate)

CHAPTER 1

INTRODUCTION

1.1. Background of the study

The ever-increasing demand for organ transplants has led to development of tissue engineering which is aimed at the growth of anchorage dependent cells of specific tissue on a scaffold. The scaffold provides the three dimensional (3d) architecture, physical support and acts as an adhesive substrate for seeded cells during *in vitro* culture towards regeneration or restoration of organ functionality (1). Such scaffolding materials must be non-toxic and non-immunostimulating to the cells at molecular level (2). Hence, the choice of the scaffolding material and its composition plays a crucial role in the success of this technique.

Considering the transplantation aspect of 3d cultures, metals, inorganic ceramic materials such as tricalcium phosphate and hydroxyapatite, synthetic polyesters such as polyglycolic acid (PGA), polylactic acid (PLA), their copolymers poly lactic-co-glycolic acid (PLGA), polycaprolactone (PCL) and natural polymers like collagen, glycosaminoglycan, starch, chitin and chitosan have been tried and tested as tissue engineering scaffolds. Metals are non-degradable and their processability is very poor. Though ceramic scaffold mimics bone components, it cannot be used for the growth of softer tissues like nerve, tendon, muscle tissue etc. These materials are inherently brittle and lack essential mechanical properties (3). Thereby, synthetic and natural biodegradable polymers are an attractive alternative due to their versatile applications in the growth of most soft tissues. Natural polymers, such as collagens, glycosaminoglycan, starch, chitin and chitosan are being tried as scaffolds to repair nerves, skin, cartilage and bone (4). Although natural biomaterials simulate the natural cellular environment, their poor mechanical properties (e.g. collagen, chitin) make them less amenable as tissue engineering scaffolds for transplantation (5).

Apart from transplantation, application of cell / tissue culture *in vitro*, involves cultivation of viruses. Major success in the control of viral diseases, such as poliomyelitis, measles, mumps and rubella, is acquired through the use of safe and effective vaccines prepared in primary cell cultures (6). 3d cultivation of cells has been used in cultivation of viruses, especially when a system close to *in-vivo* has to be experimented, like pathogen host response and immune response of host when infected by a pathogen. The methods, which

have been used to obtain the 3d culture for virology, include organotypic explants cultures, stationary or rotating micro-carrier cultures, multicellular spheroid cultures, rotating wall vessels (RWVs) or microgravity bioreactors, organotypic epithelial raft cultures, etc. All these methods require tedious procedure, very efficient technologists and dedicated time to get established. As the mode of achievement of the 3d architecture varies according to the requirement, the search for novel naturally occurring scaffolding materials with right balance of mechanical strength and biocompatibility is still underway. In this scenario, we focused our attention to an easy to use membrane based scaffold system to establish the same.

PHAs are a class of naturally occurring polyesters of various hydroxyalkanoates that are synthesized by many gram-positive and gram-negative bacteria. The formation of poly (3-hydroxbutyrate) (7) inside bacteria was reported for the first time in the last century by Lemoigne (8). Since then research in this field has developed considerably with the discovery of many more different types of organism. This polymer is accumulated intracellularly to levels as high as 90% of the cell dry weight under conditions of nutrient stress and acts as a carbon and energy reserve (9). Although the polymer is synthesized biologically, it has a very close resemblance to some of the synthetic polymers used in various fields including those used in medical applications. Due to the low availability and high production cost the polymer has remained largely unexplored.

Polyhydroxyalkanoates are commonly composed of β-hydroxyl fatty acids where the R group changes from methyl to tridecyl. More than 100 monomers have been reported and the list continues to grow. PHA can be classified into short chain length (SCL) consisting of C3 to C5, medium chain length (10) consisting of C6 to C16 and long chain length (LCL) consisting of more than C 16 poly hydroxyalkanoate (11). Among the three classes the majority are R (- 3 hydroxyalkanoicacid monomers ranging from C3 to C14 carbon atoms with saturated or unsaturated and branched or straight chain containing aliphatic or aromatic side chain (12). Poly (3-hydroxy butyrate) (PHB; R = CH3) the first identified and the most investigated PHA belongs to MCL. There are also several reports of PHA consisting of both SCL and MCL monomer units (SCL-MCL-PHA) such as poly- (3 hydroxybutyrate co- 3-hydroxy octanoate). These highly crystalline, thermoplastic polymers exhibit optical activity, isotacticity, and piezoelectric properties. Further, they are biodegradable, non-toxic, and elicit minimal inflammatory response in vivo. The copolymers degrade faster than the homopolymers. Under aerobic conditions the polymer

degrades into carbon dioxide and water. The PHBs are soluble in common organic solvents and can be processed into membranes, fibers, or microspheres. Due to their biocompatibility, processability, and degradability these polymers have been investigated as matrices for drug delivery applications and tissue engineering (13).

Owing to the variable composition of PHAs, implants made of them can have different physiochemical properties and degrade at a tailored rate in biological media, retaining their mechanical strength for a given short or extended period of time. The disparity in the properties of various PHAs arises because of their chemical composition, either from the length of the pendant groups, which extend from the polymer backbones or from the distance between the ester linkages in the polymer backbones. For the past few decades, polymers of the PHA family are paving the way for the development of new biomedical products.

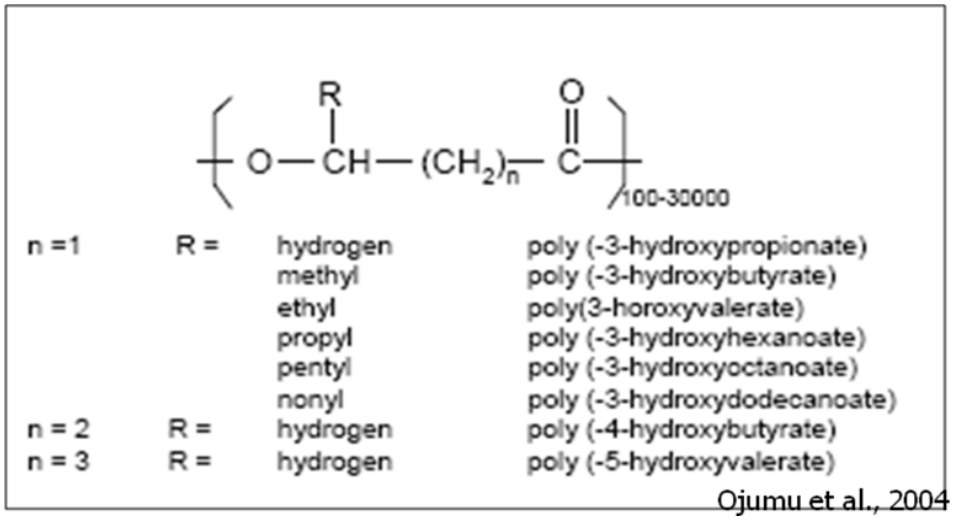


Figure 1:- **Structure of PHA.** Polyhydroxyalkanoates are composed of β-hydroxyl fatty acids where the R group changes from methyl to tridecyl (14).

As the cost of production is the major concern, research over the years has focused on the use of cost effective substrates, novel methods for extraction, genetically enhanced species and mixed cultures with a view to make PHAs more commercially attractive (8). Enormous advances in molecular analysis of PHA biosynthesis genes and PHA production have been well documented (15). A lot of strategies such as homologous or heterologous gene probes, short consensus oligonucleotide hybridization or PCR techniques have been employed for identifying PHA synthase genes and other genes involved in PHA biosynthesis (16).

Earlier reports have shown that polyhydroxybutyrate (7), a short chain length PHA (SCL-PHA) is biocompatible to various cell lines, including osteoblastic, epithelial cell and bovine chondrocytes (17). However, these polymers have also been reported to induce prolonged acute and chronic inflammatory responses (18). Hence, some researchers have tried using Short chain length- Medium chain length (SCL-MCL) co-polymers which posses superior material property behavior to overcome this shortcoming but very few studies on their biocompatibility are available so far (19).

The present study was aimed at validating one such SCL-MCL copolymer (Polyhydroxy propionate co-poly hydroxy ocatadecanoate co-polymer) produced by *Pseudomonas* sp. MNNG-S as a prospective scaffolding biomaterial for continuous and primary corneal limbal stem cell culture. Apart from this, the study evaluates the potency of the polymer in favoring viral cultivation (HSV -1) in comparison with other commercially available polymers.

1.2. Gap in existing research

With the advances in chemistry and material science, a vast array of novel synthetic polymers has been introduced over the past century. The properties like durability and strength which make the synthetic polymers attractive cause greater environmental hazards too. This brings focus towards biological polymers which are biodegradable. Among the candidates of biodegradable polymers Polyhydroxyalkanoates is considered the most suitable, because of its similar material properties as that of synthetic polymers. Cost of production of the polymer was a limitation that could be managed with newer methods of extraction which has been described recently in literature. However, the application of PHA has not been extended in many areas like the application of cell culture.

1.2.1. PHA in corneal limbal stem cell cultivation

Limbal stem cell transplantation is the method of management in corneal limbal stem cell deficiency, where the autologous Corneal limbal epithelial stem cells are cultivated, and differentiated *ex vivo* for transplantation. HAM is generally used as a carrier membrane because of its advantages of containing growth factor, anti inflammatory and anti angiogenic factors which prevents and decreases fibrosis in the healing tissue. But transparency and threat of infections are the major limiting factors associated. Moreover the use of feeder layer and complex medium containing growth factors adds on to the

safety issue. The cultivation of LESCs using animal-material free medium on HAM and on lens capsule have been recently described for transplantation purposes. Research in this field is still progressive, as there is a need for a better scaffold which is biocompatible, biodegradable and non immunogenic (20), (21), (22)).

1.2.2. Role of PHA in virology

In 3d cell culture, cells attach to one another and form natural cell-to-cell attachments. The extra cellular matrix, which the cells synthesize and secrete in three dimensions, allow systematic perturbations of the microenvironment that may result in prolonged cellular viability or expression of host proteins that are critical for viral propagation. Various approaches have been employed for 3d culture in virology. All these approaches are time consuming and laborious. A better rapid 3d culture technique would be promising in viral culture which would allow multiplication of the virus in a system closer to *in vivo* environment.

To date, identification of 3d model systems that promote desired cellular and virological phenotypes has largely been experiential. Rapid optimization of these models will benefit from the areas which allow expression of host innate immune molecules that are vital for viral dissemination. Toward this goal, this study compares some existing commercially available 3d scaffolds with PHA. Even though there are emerging commercial gel based 3d culture systems now, the possibilities of a solid scaffold like PHA in viral cultivation has not been explored.

1.3. Hypothesis

1. Polyhydroxyl alkanoate based polymer would support cellular growth, differentiation and proliferation for continuous and corneal limbal cell cultivation.

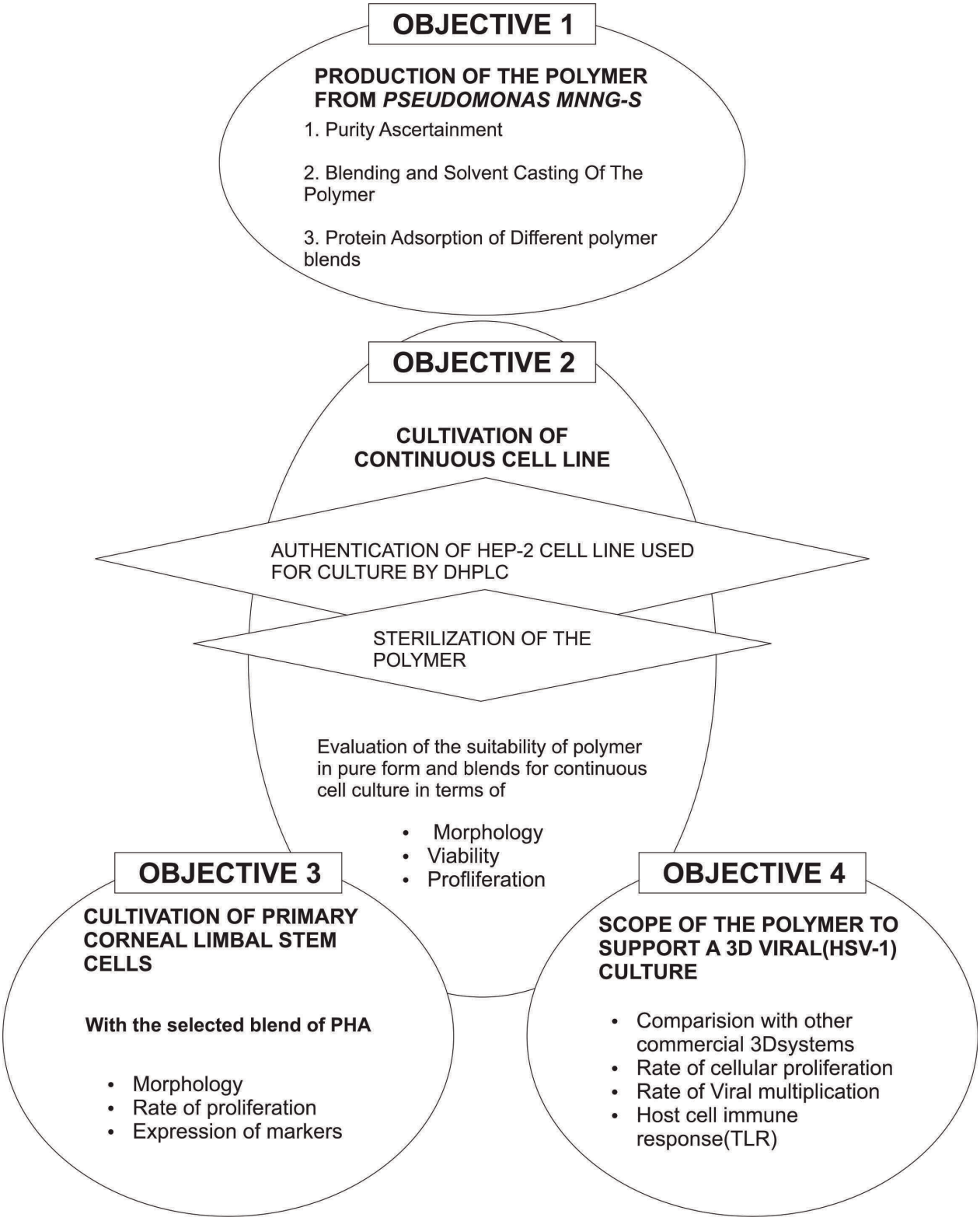
2. Polyhydroxyalkanoate based polymer would create a better cell culture system for viral studies as it may allow cells to differentiate and stratify on the scaffold.

1.4. PHA used in the current study

In this study, poly hydroxypropionate co-poly hydroxy octa decanoate polymer, which is an SCL-MCL copolymer, produced from *pseudomonas MNNG-S* was explored for its suitability in cell and viral culture.

1.5. Objectives

The overall objectives and work flow of the study are listed in the flow chart.



CHAPTER 2

REVIEW OF LITERATURE

2. 1. Cultivation of cells in twsxdd4o dimensions (2d) versus three dimensions (3d)

The traditional cell culture which is 2d in nature provides a little physiologic resemblance of the growth environment *in vivo* with respect to the cellular morphology, cell-cell and cell matrix interactions, leaving a gap in monolayer cell culture and whole animal. The 3d *in vitro* culture has emerged as an approach that mimics the *in vivo* cell growth environment (23).

Cells grow on flat plates or dishes, made of surface modified plastic, constitute 2d culture. As the cells adhere and spread on flat surface, it forms unnatural cell to cell and cell to surface attachment. Protein secreted by the cells also may get denatured, as the cellular adherence is not natural .Whereas, in 3d cell culture, cells adhere to each other with the support of extracellular matrix (ECM), which the cells secretes, and forms natural cell-cell interaction. The ECM is composed of various proteins in their native configuration. As the cells are in close proximity, it exerts forces on one another to help them in migration(24), (25). The 3d culture system provides a well defined geometry and exhibit phenotypic and functional similarity to *in vivo* environment.

At present, research on 3d cell systems is more vital and productive than ever before, as the potential of 3d cell cultures is being exploited in many areas of biomedical research. The method to achieve the 3d culture varies according to the requirement. A wide variety of natural and synthetic polymers have been evaluated for the possibility to give 3d architecture of cells.

2.2. Polyhydroxyalkanoates

PHAs are a versatile class of biopolymers produced by some bacteria under unfavorable growth conditions.

2.2.1. PHA producers

The first observation of accumulation of PHA in bacteria was by Beijerinck in 1888 (26). Poly hydroxyl butyrate was the first polymer to be determined and was considered as of P(3HB) hydrolysis (27). The requirement of limiting conditions (with a high carbon to

nitrogen ratio) of the bacterial growth for the production of PHA was noticed in 1958 by Macrae and Wilkinson. He observed that P(3HB) was stored when the medium had a high C:N ratio and it was concluded that P(3HB) is a intracellular reserve material forms under unbalanced growth condition (28). Later it was reported that PHA production can occur at non limiting conditions also by certain bacteria. Now more than 300 species of bacteria are known to produce PHA (28, 29). Most of the PHA producing bacteria belongs to the phylum *Proteobacteria*, which mostly includes *Eubacteriacea* (30).

The growing interest in PHA results in extensive research to identify new species of bacterium or other PHA producers. A lot of bacteria like *Comamonas tetosteroni, Clostridium butyricum ATCC 1938, Nostoc muscorum, Rhodospirillum rubrum, Synechocystis sp*. have been tried for their capability as PHA producers cultured with various substrates (31, 32), (33). *Aeromonas caviae*, isolated from soil accumulates PHA from alkanoic acids and plant oils (34). *Pseudomonas* is the most studied MCL PHA producing bacteria while *Cupriviadus necater* is the most studied SCL-PHA producer. A strain of *pseudomonas* capable of producing SCL-MCL copolymer named *Pseudomonas LDC-5* was identified by Sujatha et al (35) and a mutant strain MNNG-S has been created for increased production of the copolymer (36). A tremendous advance in molecular analysis of PHA biosynthesis also has been documented (37, 38). Strategies like short consensus oligonucleotide hybridization, homologous or heterologous gene probes, or PCR techniques have been employed for identifying PHA synthase genes and other genes involved in PHA biosynthesis (39, 40). PHA producers are also found among *Cyanobacteria (Synechococcus), Firmicutes (Bacillus*), etc. Strains which produces PHA have been isolated from various environments including activated sludge (41-43), marine waters (44), hypersaline environments and oil contaminated soils.

2.2.2. Biosynthesis of PHA

PHA s, commonly used for biological application, can be divided into two main classes, SCL and MCL. PHB and PHV belong to SCL class of PHA and PHO (poly 3-hydroxy octanoate) belongs to MCL class. Four different pathways have been reported so far for the biosynthesis of PHA (12), (45),(46).

2.2.2.1. Biosynthesis of SCL PHA

Primary P (3HB) biosynthetic pathway consists of 3 main reactions catalyzed by three discrete enzymes as shown in Figure 2(47). The initial step is a condensation reaction of two molecules of acetyl-CoA to form acetoacetyl-CoA with the help of the enzyme β -ketothiolase (*phaA*) followed by the reduction of acetoacetyl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase *(phaB*). Then the (R)-3-hydroxybutyryl-CoA monomers are polymerized into P (3HB) by P (3HB) synthase or P(3HB) polymerase *(phaC*). PHA synthase is the key enzyme of PHA biosynthesis. The pathway has been studied in detail in *Ralstonia eutropha*.

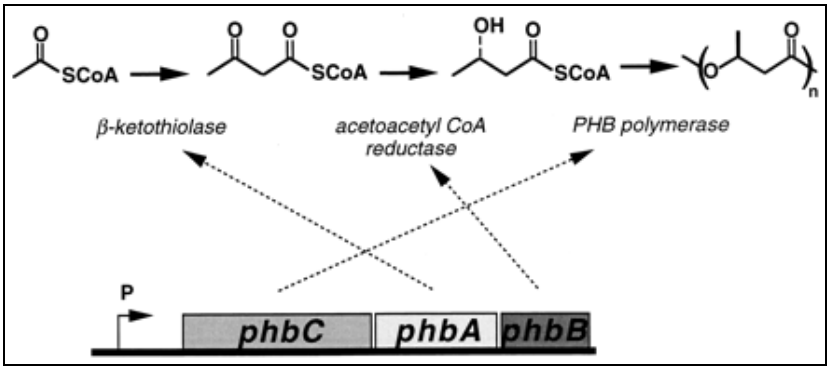


Figure 2:- Biosynthetic pathway for P(3HB). P(3HB) is synthesized in a three-step pathway by the successive action of β-ketoacyl-CoA thiolase (7), acetoacetyl-CoA reductase (*PhbB*), and P(3HB) polymerase (*PhaC*) (47)**.**

2.2.2.2. Production of MCL PHA

The comparison of MCL PHAs formed by *pseudomonas* is directly related to the structure of carbon source (47, 48). **Figure 3** shows different pathways which depend on the source of carbon.

2.2.2.2.1. Chain elongation reaction

Chain elongation reaction occurs in PHA synthesis from hexanoate where acetyl-CoA moieties are condensed to 3-hydroxyacyl-CoA. Ketoacyl-CoA forms from acetyl -CoA when acyl -CoA added to it. Ketoacyl-CoA reductase then converts ketoacyl -CoA to (-3-OH-acyl-CoA (49).

2.2.2.2.2. Beta oxidation pathway

When fatty acids are used as carbon source, PHA production follows β-oxidation pathway. Fatty acids are taken up by the bacteria and degraded, through removal of C2 unit as acetyl-CoA, followed by oxidation of acyl-CoA to ketoacyl-CoA.The pathway then proceeds by the oxidation of acyl-CoA via 3-hydroxyacyl-CoA intermediates. Formation of (R)-3- hydroxyacyl-CoA from intermediates is catalyzed by one of the three enzymes, enoyl-CoA hydratase (PhaJ), 3- hydroxyacyl-CoA epimerase or 3-ketoacyl-CoA reductase (FabG). The last step is the polymerization of (R)-3-hydroxyacyl-CoA by PHA polymerase (50).

2.2.2.2.3. Fatty acid denovo synthesis

When growth depends on carbon sources like gluconate, acetate or ethanol which are metabolized to acetyl-CoA., the intermediates are derived as (R)-3-hydroxyacyl-ACP after a number of degradation steps. This (R)-3-hydroxyacyl-ACP is then converted to (R)-3-hydroxyacyl-CoA through an (R)-3-hydroxyacyl-(ACP to -CoA) transferase (PhaG) which forms a link between β-oxidation pathway and fatty acid biosynthesis (51)**.**

2.2.2.3. Production of SCL- MCL co polymer

Some *Pseudomonas* spp. can incorporate both SCL and MCL PHA monomers in the same polymer chain (47), when grown on unrelated carbon source such as carbohydrates, 1,3-butanediol, glucose or gluconic acid (52). It has been reported that both the β-oxidation and de novo fatty acid biosynthesis can function concurrently in the synthesis of PHA (53).

2.2.3. Genes responsible for PHA production

Gene responsible for the production of PHA varies in various microorganism as the enzyme differs in production of SCL and MCL PHAs (9). Genetic organization of different genes encoding various classes of enzymes are shown in **Figure 4**. In SCL biosynthesis *phaA* (β-ketothiolase), *phaB* (acetoacetyl-CoA reductase) and *phaC* (*PHA synthase / PHA polymerase*) genes are involved, whereas, MCL PHA uses added genes called *phaG* (3-hydroxyacyl-acyl carrier protein-coenzyme A transferase) and *phaJ* (enoyl-CoA hydratase) (29). *PHA synthase* gene also differs in different strains of bacteria. *Pseudomonas* strains have 2 different *phaC* genes, *phaC1* and *phaC2* which differs in substrate specificity and monomer composition (54) of the PHA accumulated. When the

limiting nutrient is restored, a gene called *phaZ* (PHA depolymerase) muster the PHA granules by liberating carbon and energy.

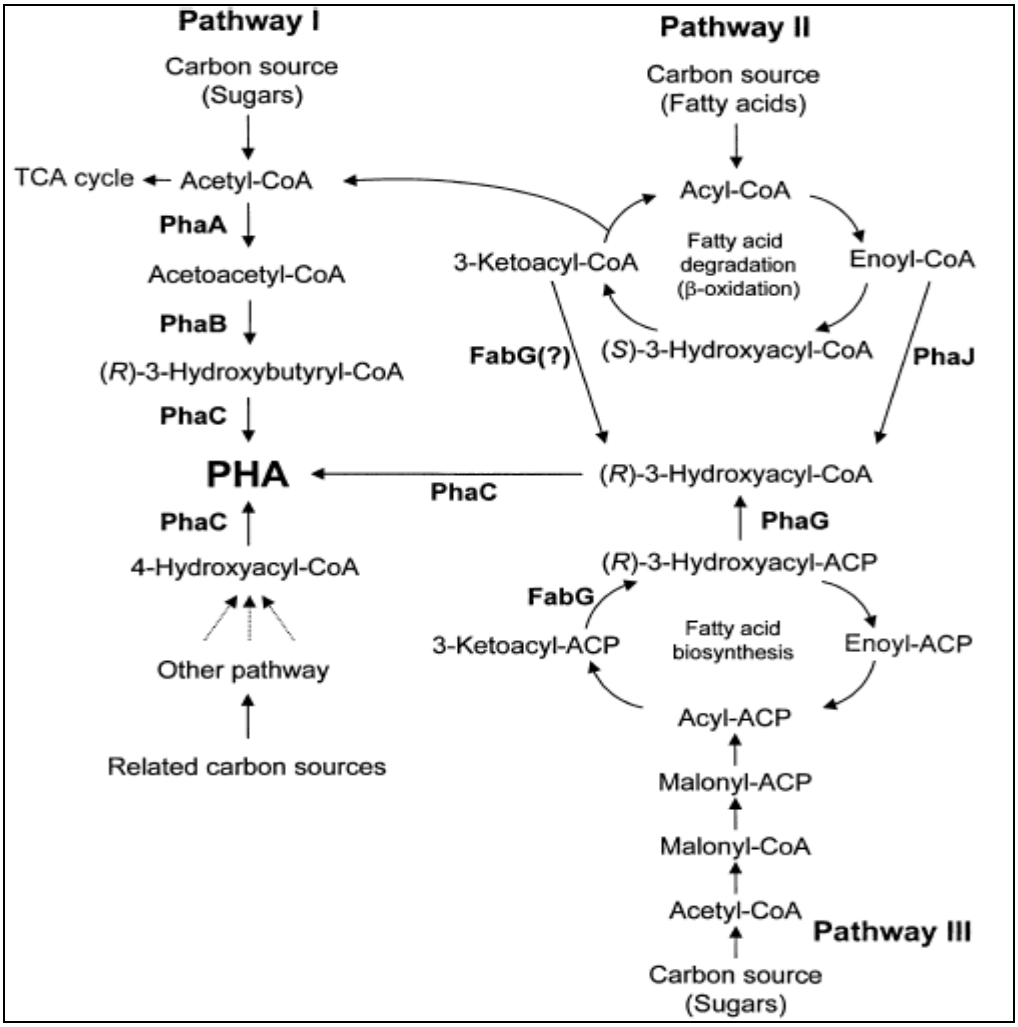


Figure 3:- Metabolic pathways that supply various hydroxyalkanoate (HA) monomers for PHA biosynthesis. PhaA, 3-ketothiolase; PhaB, NADPH-dependant acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ, (R)-specific-enoyl-CoAhydratase; FabG, 3-ketoacyl-ACP reductase; Source: (55).

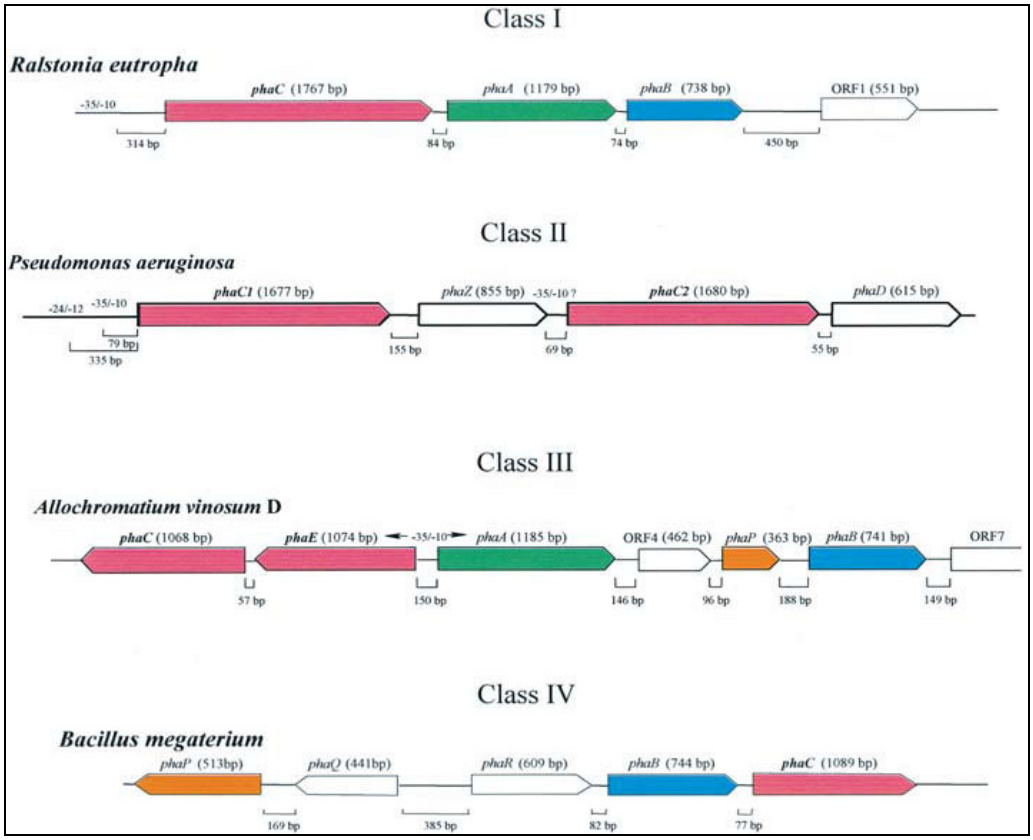


Figure 4:- Genetic organization of representative polyester synthase genes encoding the various classes of enzymes. PhaC/C1/C2, gene encoding PHA synthase; *phaE*, gene encoding subunit of PHA synthase; *phaA,* gene encoding β-ketothiolase; *phaB*, gene encoding acetoacetyl-CoA reductase; *phaR*, gene encoding regulator protein; ORF, open reading frame with unknown function; *phaZ*, gene encoding PHA depolymerase; *phaD*, open reading frames with unknown function. Source: (56)

2.2.4. Biogenesis of the PHA granule:

In literature, two models of PHA granule have been described: (i) the micelle model and (57) the budding model (30). In both of the models, PHA granule formation begins with availability of the substrate which is (R)-3-hydroxyacyl-CoA thioesters. The PHA synthases act upon these esters and polyester chain starts forming which remains covalently attached to the enzyme. The growing polyester chain forms amphipathic molecules self assemble and form water insoluble inclusions with an amorphous polyester core and PHA synthase attaches covalently to the surface. These granules increase in size with the PHA synthase incorporating more molecules till maximum PHA accumulation is achieved. Usually 5 to 8 PHA granules are deposited intracellularly. The PHA granules are surrounded by a phospholipid membrane with embedded or attached proteins.

2.2.5. Detection of PHAs

2.2.5.1. Detection of PHA synthase gene

The strategies which have been useful in identifying the genes responsible for PHA synthesis include PCR, short consensus oligonucleotide hybridization, homologous or heterologous gene probes (39), (40). There are a number of primers which have been designed and tested for detecting different classes of PHA synthases (58, 59), (60).

2.2.5.2. Detection of PHA granules

The detection of PHA granules is usually done with the help of staining with dyes. The earlier used dyes like Sudan Black B (61), a water soluble basic oxazine dye, Nile Blue A (62) and Nile Red which is an oxazone form of Nile blue A needed post staining at the expense of the culture. However, it was Spiekermann and her colleagues who showed that PHA can be detected directly in the growing bacterial colonies by simply adding Nile Red into the medium without inhibiting bacterial cell growth (63). Using the Nile Red containing medium PHA accumulation in the cells can be estimated at any given time point during the experiment. The **Figure 5** shows accumulation of PHA at 48 hours of cultivation in *pseudomonas MNNG S,* which is used in the current study.

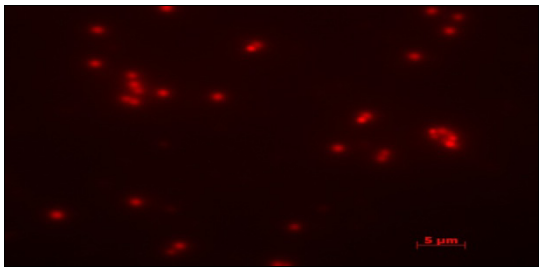


Figure 5:- Accumulation of PHA. PHA accumulation in *Pseudomonas* at 48 hours of culture. PHA granules are stained with Nile Red.

2.2.6. Isolation of PHA granules

A number of methods are available for PHB and PHAs extraction. Isolation of the PHA granules was attempted first by repeated centrifugation of DNase-treated bacterial cell extracts layered on glycerol to collect the granules at the interface. Crude PHA granules isolated from *Zoogloea ramigera* by sonication, sucrose density gradient centrifugation were able to retain their activity for 6 months when stored at -20 ^{0}C.

Now, among a variety of extraction methods, the two main approaches are cell disruption followed by aqueous extraction and organic solvent extraction. Both the methods have certain advantages and disadvantages. Organic solvent extraction is more commonly used with a better recovery yield though it is more expensive (14). The organic solvents used are chloroform, methylene chloride, propylene carbonate or dichloroethane. The first step consists of refluxing with these solvents; the resultant solution is then filtered and concentrated; and the polymer is precipitated with methanol or ethanol.

2.2.7. Analysis of PHAs

Different methods are available for determining the PHB / PHA content, the composition of PHAs and the distribution of monomer units. Spectrophotometry was the first method and it involved conversion of the polymer to crotonic acid by heating with concentrated sulphuric acid before spectrophotometric analysis (64). Chromatographic methods like Gas chromatography (GC) and High-pressure liquid chromatography (HPLC) took the place of spectrophotometry with mild variations in sample processing (65). This method was later improved by using propanol and HCl. Nuclear Magnetic Resonance (NMR) technique has also been applied to characterize PHAs. A number of NMR studies for different PHAs to determine the constituent monomers have been done (66). It has also been shown that Fourier Transform Infra Red spectroscopy (FTIR) can be used for determining the crystallinity of polymer (67). Scanning, Phase Contrast and Transmission Electron Microscopy have also been done to get an idea of the morphology of these granules within the cell (59, 68).

2.2.8. Factors affecting PHA production

The important factors, which have to be taken care of, to get a good yield of the polymer in a cost effective manner include the selection of microorganism. The ability of the microorganism to utilize an inexpensive carbon source for efficient production has to be preferred. Faster growth rate and developing copolymers blends by using engineering recombinant microbes such as *E.coli*, *P. oleovorans*, is also being investigated (9, 28, 29). Growth rate, polymer synthesis rate and the maximum extent of polymer accumulation of a particular cell is based on the substrate (14).

It is the medium used which determines the nature of the polymer, whether it is homo polymer or co polymer. Some of the sources which are deeply studied to get a cost

effective production of PHA are swine waste (69), starch and starchy waste waters effluents from olive mill and palm oil mill (70), molasses (71), etc. The mode of fermentation is another factor which influences the productivity of the polymer. Several methods such as Fed-batch and continuous cultivations have been carried out to improve productivity (5, 12), (36).

The mode of recovery of PHA also has a major role in bringing down the production cost. Pretreatment of cells, cell disruption, chemical or enzymatic digestion of non-PHA in the system, spontaneous liberation of PHB, dissolved air flotation and extraction using supercritical CO_2 have been tried to increase the PHA productivity and reducing the overall cost (72).

2.2.9. Biological degradation of PHAs

PHAs undergo degradation with the help of microorganisms which colonize on the surface of polymer. The microorganism takes this polymer as energy source and secretes enzymes to degrade. These enzymes include PHA hydrolase and depolymerases (47). PHAs are degraded aerobically into water and carbon dioxide, while methane is produced in anaerobic conditions.

The ability of the polymer to be degraded has been evaluated by monitoring their properties like molecular weight, strength and dimension. The rate of polymer degradation depends on a variety of factors including surface area, composition of the polymer, physical form (amorphous or crystal, microbial activity of the disposal environment, pH, temperature, moisture and the presence of nutrient materials (42). Copolymers with P (3HB) polymers are degraded more rapidly than either P alone or P(3HB-co-HV) copolymers (9). The degradation rate of a piece of P (3HB) is typically on the order of a few months in anaerobic sewage to almost a year in sea water (47).

The biomedical applications of PHA relies on its complete biodegradability *in vivo* after the success of many *in vivo* experiments (73). *In vivo* experiments for studying biodegradability of PHAs were conducted by Korsatko in mice and observed that PHB of molecular weight greater than 105 was undesirable for long term medication dosage (7). Miller and William s also carried out studies on PHB and copolymer containing 3HB and 3HV showed that PHB biodegradation occurred only when PHB has been predegraded by

10 Mrad of γ-irradiation prior to implantation. Apart from this the nonspecific esterases produced *in vivo* have a role in mediating the rate of degradation of PHB (74).

2.2.10. Production of PHA from *Pseudomonas LDC -5* mutant strain *MNNG -S*

Pseudomonas sp.LDC -5 was originally isolated from soil samples from Madurai district of Tamil Nadu, India. Among the hundreds of indigenous strains screened, Sujatha et al identified 2 species of *pseudomonas LDC-5* and *LDC-25*, using MCL-PHA PCR for the *PHA synthase* genes (75). The characteristics of *LDC-5* was studied further by cloning *PhaC* gene of the species to *E.coli* (15, 76). The growth characteristics and effect of different substrates on production of the polymer by *Pseudomonas LDC-5* and its mutant form *MNNG-S* was studied. This study showed that the mutant strain, *MNNG-S* produced higher and quicker yield compared to wild type LDC-5, amongst the minimal medium studied. Biomass and PHA yield was maximal through the use of ammonium sulphate as nitrogen source (36).

2.3. PCR methods for cell line authentication – The advent of DNA fingerprinting methods for identifying cell lines

Identifying species of origin and intra species contamination of a cell line is most important as it affects downstream applications of the cell line. Number of methods like isoenzyme profiling, karyotyping and DNA fingerprinting are available to check authenticity of cell lines. Among these, DNA fingerprinting plays major role in cell authenticity. Southern blotting was the method adopted earlier for DNA fingerprinting though gene scan based approaches took the way later.

PCR-based methods have been adopted for the detection of species of origin of the cells as well as interspecies cross contamination among cell lines. The results of isoenzyme analysis were confirmed by PCR assays in order to validate the method using a panel of 35 human and animal cell lines containing 9 species specific primers that anneal to the species specific DNA sequences. *Cytochrome b* gene is unique in each species and allows the determination of species identity. *Cytochrome b* gene is also used in PCR-RFLP method for cell line identification. In this method, a primer was allowed to anneal to a portion of *cytochrome b* gene by PCR followed by restriction digestion of the amplified product. The particular pattern in agarose gel verified the species specificity of the cell line. Both PCR – RFLP and isoenzyme were found to have more or less equal sensitivity (77). *Aldolase*

gene family is another gene which is conserved over a wide range of animals and humans. However indistinguishable pattern obtained in closely related species like human which suggested that *aldolase* gene amplification should be coupled with DNA sequencing to obtain specific results (78).

Polymorphic repeat sequences have been used to identify the source of origin within the species. Among such DNA probes, VNTRs, are used in southern blotting to identify cell lines. Use of polymorphic probes results in generating a profile for each individual in forensic application. The same is later extended to verify the identity of individual cultured cell lines collected in the Japanese cell banks, JCRB, RCB and IFO. They used a mixed probe system among which distinct bands, at least four to eight, were used for cell line authentication. These bands were widely spread in a range of molecular sizes, and were stable and reproducible under stringent conditions of southern blot hybridization (79).

The major disadvantage with above mentioned methods is that they predominantly identify only interspecies contamination. Polymorphic regions like VNTRs, RFLPs and other conserved genes like cytochrome b necessitate further use of sequencing to establish solid results. This is where STR regions gained more importance, because of their ability to distinguish between animals belonging to the same species.

2.3.1. Recommendation of STR profiling for human cell line authentication –ATCC SDO

ATCC® SDO workgroup ASN-0002 "Development of a consensus standard for the authentication of human cells: standardization of STR profiling" was formally assembled in early 2009 as a result of a proposal submitted in 2008 by John Masters and Roland Nardone (80). As per this, STR profiling was selected as the recommended authentication technology to resolve human cells to the individual level. In addition, the STR profiling technology was commercialized in a kit form as a universal reference standard technology for the authentication of human cell lines. Using the kit, 253 cell lines were analyzed from a number of sources worldwide using the technology of STR fingerprinting resulted in an accurate bar-coding of each cell line (80). A data base has been created with the STR profiles of cell line, with the issuance of the Standard, and has been continuously updated. Thus, the frequency of misidentification of human cells will be reduced by the comparison of STR profiles generated from individual cell stocks to the database (81).

2.3.2 Federal Bureau of Investigation (FBI codis) core STR loci – from forensics to cell culture

The FBI has chosen 13 specific STR loci to serve as the standard for Combined DNA Index System (CODIS) as shown in **Figure 6**. The intention of ascertaining a core set of STR loci is to guarantee that all forensic laboratories can establish uniform DNA databases in order to share valuable forensic information (82).

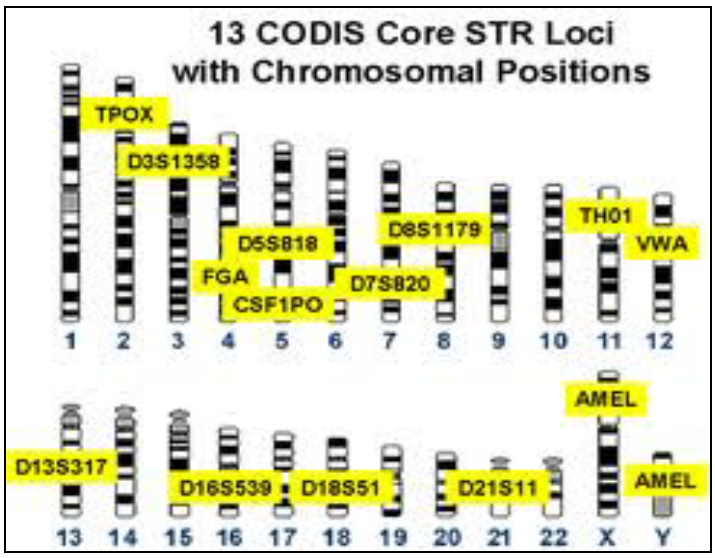


Figure 6:- Core STR loci proposed by FBI

STR profiling is mainly used in forensic studies to establish identity of culprits involved in a crime using evidence (generally fluids) found from the crime scene. This technique has been extended onto cell culture studies for authentication cell lines, especially of human origin. Considering that a majority of human-derived cell lines are obtained from different individuals, each cell line is naturally bound to have a different STR profile. This concept is utilized in cell line authentication using STR profiling. With this as basis, cell culturists have started developing STR profiles for cell lines of other species like rat, mouse and rabbit. It is important to understand that STR profiling is useful in identifying intra-species cell line contamination only, as identification is on a sequence level, as opposed to cytogenetic studies, where identification is on a chromosomal level.

Methods for STR fingerprinting

Separation by PAGE

STR profiling predominantly employs PAGE for visualizing amplified STR loci products. Silver Stain Detection Kit developed by Promega was used in conjunction with PAGE for STR profiling of human epithelial cells from buccal swab. Three specific STR loci (CSF1PO, TPOX, and THO1) were amplified by PCR and PAGE was used to separate allelic bands associated with the STR regions. Silver nitrate, the prime chemical in silver staining method, can react with chloride residues found in the fingerprint and forms silver which reveals a black or reddish-brown fingerprint under UV light (83).

The National Cell Bank of Iran (NCBI), with the above procedure as the basis, utilized the 13 CODIS- based STRs along with gender determination gene to establish a reproducible approach for authentication of 100 human cell lines. Among the NCBI cell lines, 18.8% were found to be cross-contaminated (84).

2.3.4. Isolation of STR regions using DHPLC

DHPLC is based on a reversed phase system in which the stationary phase is nonpolar and the mobile phase polar. The hydrophobic stationary phase, DNASep column marketed by the company Transgenomic, is made up of alkylated nonporous poly (styrene-divinylbenzene) particles 2–3 μm in diameter. The polar mobile phase is acetonitrile (CH3-CN). However, DNA molecules are large anions because of the negative charges on the phosphate groups in the phosphate-sugar backbones of the DNA strands.

Organic cations are required to allow interaction between DNA anions and the nonpolar stationary phase. The organic cation carries a positively charged portion to interact with the negative charge of DNA molecules on the one hand, and also a hydrophobic portion to interact with the nonpolar stationary phase on the other hand. The most commonly used organic cation is triethylammonium, (CH3CH2)3N+, in the form of triethylammonium acetate (TEAA). Thus, TEAA is used as an ion pairing reagent. The triethylammonium cations bind to the phosphate groups of DNA molecules and hence effectively coated the DNA molecules with a hydrophobic layer (the triethyl portion).

The number of TEAA molecules coating the DNA molecules is proportional to the length of the DNA molecules and in turn determines the degree of interaction between the DNA

molecules and the stationary phase. DNA molecules are eluted from the column in an increasing gradient of acetonitrile (buffer B), which weakens the interaction between coated DNA molecules and the stationary phase. In other words, coated DNA molecules bind onto the stationary phase and will be released from the stationary phase when acetonitrile in the mobile phase reaches a specific concentration. Thus, shorter DNA molecules are eluted earlier from the column and hence separated from longer DNA molecules under the same buffer condition. In summary, the separation of DNA molecules is based on the principle of ion-pair reversed phase liquid chromatography.

Three modes of operation are available for DHPLC, depending on the temperature of the column.

- Non-denaturing condition – It is applied to the size dependant separation of double stranded DNA molecules. The column temperature is maintained at 50 ^{0}C. The concentration of the eluent (acetonitrile) is increased with time. The shorter DNA fragments are eluted first, followed by longer DNA fragments. The eluted fragments are detected by UV detector and the chromatographic peaks representing the corresponding DNA fragments are displayed in the screen.
- Partially denaturing condition – It is applied for screening unknown mutations and putative SNPs. The column temperature is maintained between 50 ^{0}C- 70 ^{0}C. The PCR product from a test sample is mixed with a homozygous reference PCR product in equal volume. The mixed DNA fragments are denatured and allowed to re-anneal by gradually lowering the temperature, forming homoduplexes and heteroduplexes. Heteroduplexes with mismatches are less stable and are thus eluted before homoduplexes. With reference to a homozygous wild type control, any difference in elution profile is indicative of sequence variation.
- Completely denaturing condition – This mode can differentiate between single stranded DNA (and RNA) with separation depending on length and base composition. It can be used to analyze and isolate RNA and synthetic oligonucleotides. The column temperature is maintained between 70 ^{0}C-80 ^{0}C (85).

The WAVE system developed by Transgenomic, was used to analyze the FI3A01 STR locus at human chromosome 6p24-25 by separation and purification with peak capture by DHPLC. The allelic range of these loci is from 3 to 20 repeats and the repeat sequence is AAAG. To analyze the nature of the peak capture method for PCR products, three

fractions were captured at 18s intervals. While two fractions show peaks corresponding to individual alleles, the third fraction demonstrates the presence of both alleles. The fractions containing individual alleles were subject to sequencing by capillary electrophoresis with accuracy greater than 96% (86). In a study conducted in 2003, size-based separation of DNA fragments using DHPLC was carried out. Non-denaturing conditions were employed for separation and purification of STR alleles with a good resolution (>1) in less than 14 minutes (87).

2.4. Corneal limbal stem cell culture and transplantation

2.4.1. Surface of the eye

Cornea conjunctiva and lacrimal system constitute the ocular surface. Ocular surface is different from other body surface as it has to maintain the optical clarity by hydrating cornea and conjunctiva and protect the globe from mechanical, toxic and infectious trauma. Cornea is the most unique one on ocular surface in being both avascular and transparent in order to transmit the light to retina. According to the World Health Organization, corneal disorders, constitute a significant cause of blindness in the world (88).

The cornea is composed of five layers as shown in **Figure 7**. Corneal epithelium which forms 10% of the total corneal thickness absorbs nutrients and oxygen and protects the eye. The epithelium consists of basal cells, wing cells and squamous cells. Corneal stroma is a connective tissue which takes 90% depth of the cornea. It is composed of collagenous lamellae consists of tightly packed collagen fibrils embedded in a hydrated matrix of glycoproteins and proteoglycans. Towards the anterior stroma, below the epithelial basement membrane is bowmen's layer which is acellular. Desciment's membrane, the basement membrane for corneal endothelium is the posterior layer of the cornea which pumps excess water out of stroma, prevents corneal edema and maintains corneal transparency (89).

2.4.2. Corneal limbal epithelial stem cells

As corneal epithelium is the outermost protective layer of the eye it needs to withstand the attrition caused by the light induced damage and dryness induced by exposure. To cope with this, it has a capacity of self renewal which is governed by corneal epithelial stem cells residing at corneal limbus. Corneal limbus as shown in **Figure 8** is the outer vascular rim at the junction of cornea and conjunctiva. When cornea damages, due to burns or

injuries and several other diseases, stem cells residing at the limbus are induced to proliferate in order to support the growth of new epithelial cells over the surface. This natural recovery process is impaired when the ready source of limbal stem cells too is damaged, and this condition is called limbal stem cell deficiency (LSCD).

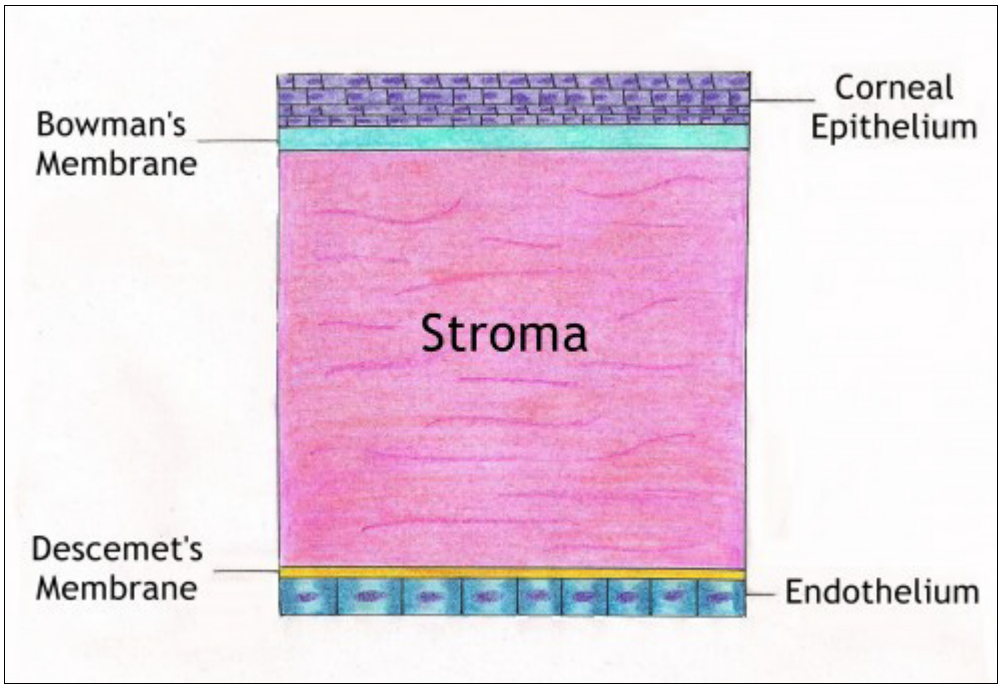


Figure 7:- **Different layers of cornea.** Cornea comprises of 5 layers, Epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium.

2.4.3. Corneal limbal stem cell transplantation (LSCT)

Limbal stem cell transplantation is at present the method of management adopted for those patients with LSCD. Autologous limbal stem cell transplantation particularly has a better clinical outcome where limbal tissue for transplantation is from the unaffected fellow eye, than allo-transplantation, where the limbal tissue is collected from related or non related donor eye. Possibility for the occurrence of limbal stem cell deficiency at the donor site over several years is a major concern related to autologous limbal stem cell transplantation. To overcome this issue, reconstruction of corneal epithelium by tissue engineered corneal epithelium has been studied extensively (10, 90), (91).

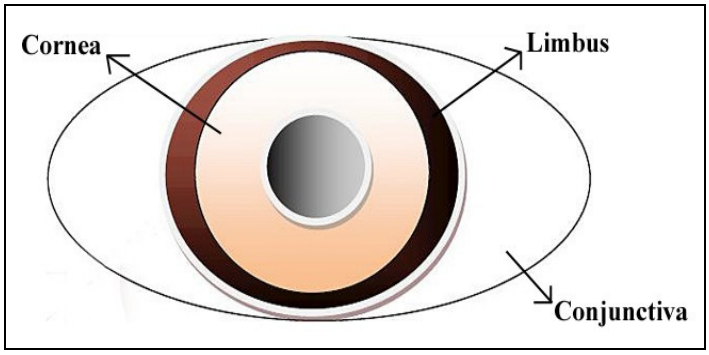


Figure 8:- **Ocular surface**. Surface of the eye consists of cornea, limbus and conjunctiva.

This is achieved by the expansion of corneal epithelial cells *in vitro* from a small limbal/corneal progenitor population of cells on a carrier or scaffold followed by transplantation of the cultured cells with or without the carrier. Even though the success is not always attained, in some cases the early clinical outcome appear promising mainly because immune mediated rejection is avoided (92).

Although human amniotic membrane is the common carrier for corneal stem cell culture various natural as well as synthetic materials have also been studied as substrates. These include extra cellular matrix (ECM) proteins such as collagen types I, III and IV, fibronectin, laminin, gelatin, vitronectin, thermo reversible polymers and PHEMA (93), (94).

2.5. Scope of 3d culture in virology

Animal virology evolved as a laboratory science between 1948 and 1955 with the involvement of 4 important stages. (i) Sanford et al. at the National Institute of Health (NIH) overcame the difficulty of culturing single cells (57). George Gey and his colleagues at John Hopkins Hospital cultured and passaged human cells for the first time and developed a line of cells (HeLa) derived from a cervical carcinoma (iii) Harry Eagle at the NIH developed an optimum medium for culturing single cells and in 1949, John Enders, Thomas Weller and Frederick Robbins (93) found out that poliovirus could multiply in cultures of non neuronal human cells. The two instant effects of these radical findings are the development cell culture based viral vaccines, such as polio, which was the first cell culture based vaccine, rubella, measles, etc and the development of a system for studying the biochemistry and molecular biology of viral replication to begin the modern era of molecular virology (95). As the cells in 2d could not satisfy the real scenario of viral

infection many strategies have been adopted to create a 3d cell culture for cultivation of viruses.

2.5.1. 3d cultures using tissue or organ explants

The development of organ explants using mucosal tissues was a breakthrough in the research of HIV infection as the transmission of virus occurs across the mucosa. a culture model developed from cervical squamous tissue was developed to show the transmission of HIV to the female genital tract (96). The experiment included trans-wells with the tissue surrounded by agarose (96). In Another study, the attachment of HIV inhibitors for their ability to prevent the infection *ex vivo* was evaluated using cervical tissue model (97).

2.5.2. 3d cell cultures using micro-carriers

Micro-carrier cultures were developed as a strategy to enhance the replication of viruses in order to produce vaccines on a large scale. Today, Vero cell line, as it is considered to be suitable for viral vaccine production, (98) is used in micro-carrier suspension cultures in bioreactors. These culture systems could achieve higher viral titers and facilitated vaccine production even the developing countries as required by WHO (99, 100). The optimization of virus yield by Vero cells in micro-carrier cultures (in spinner flasks or in a bioreactor) has been documented by several researchers (101), (102). Another successful attempt based on 3d viral culture on micro-carrier was cultivation of hepatitis C virus (HCV) *in vitro*, using hepatocytes where, it is differentiated to attain functional and morphologic similarity (103). A vertically extended cylindrical matrix containing porous glass beads are used to enhance the flow of liquid medium and the cells solidly attached on the surfaces and within the pores of micro-carriers.

2.5.3. 3d cell cultures using multicellular spheroids (MCS)

Multicellular spheroids (MCS) derived from cell lines or primary tumor cells resemble the tissue as that of *in vivo*, more closely than tumor cell lines and acts as a model of tumors. As these cultures remain in their original microenvironment without any process of *in vitro* selection, it drew attention for studying adenovirus distribution which could not be studied in 2d cultures.

The study for demonstration of HSV thymidinekinase/ganciclovir (HSV*tk*/GCV) suicide system using HSV*tk* expressing cells confirmed the importance of the MCS system (104).

This study reported that the Herpes Simplex HSVtk-expressing cells grown as MCS manifested a lower GCV cytotoxicity and bystander effect compared to the same cells grown as monolayers.

2.5.4. 3d cell cultures using organotypic epithelial raft Cultures

Schematic representation showing the different steps used in the production of organotypic epithelial raft cultures is given as Figure 9. The epithelial cells are seeded on the top of the dermal equivalents collagen matrix with feeder cells and maintained submerged for 24–48 h. The rafts are then lifted (day 0) and placed onto stainless steel grids at the air–liquid interface and fed by the medium underneath and incubated for a period of 10-14 days. Epithelial cells stratify and differentiate into full thickness.

The Organotypic Epithelial Raft Cultures permit full differentiation of the epithelial cells and accurately mimic the physiology of the epidermis. Many important human pathogens, including various viruses, target the epithelium during at least some part of their natural history. Pathogenic viruses use human epithelium as the initial site of infection, the site for replication, a staging area for transportation to other tissues, and a site for latency or persistence. The 3d raft cultures allow the cells to undergo the authentic program of differentiation, providing a unique and essential tool for the study of differentiation-dependent biology of viruses and other pathogens.

Organotypic epithelial raft cultures have proven to be a breakthrough in the research on human papillomaviruses (HPV), since their life cycle is tightly linked to the differentiation of the host epithelial tissue. This link to differentiation hampered the study of the HPV life cycle for many years.

The organotypic epithelial raft culture system, which mimics important morphological and physiological characteristics of the epithelium has proved to be a valuable tool for studying HPV (105). Applications of the organotypic culture systems to HPV-11 infected condylomatous tissue explants and to an immortalized human epithelial cell line that is derived from a cervical dysplasia and harboring episomal HPV-31b DNA have validated this culture system, in that progeny HPV virions were produced for the first time *in vitro* (106).

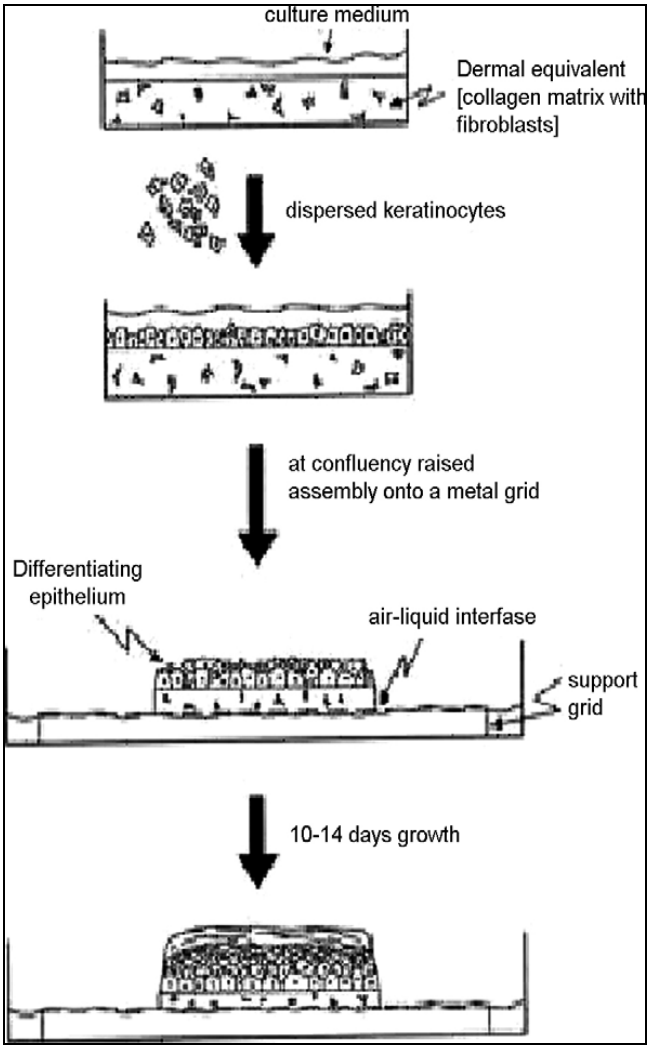


Figure 9:- Epithelial raft culture. Need of air liquid interface for epithelial differentiation. These cultures are constructed by placing the epithelial cells on top of a dermal equivalent and then raising the cells to the air–liquid interface. The dermal equivalent is composed of natural dermal elements (collagen matrix with fibroblasts) or a synthetic dermal matrix maintained on a rigid support. Normal epithelial cells stratify and differentiate into full-thickness mimicking the original tissue from which the particular keratinocytes were derived (107, 108), while cells derived from neoplasias with dysplastic morphologies, in the raft cultures appears to be similar to the pre neoplastic lesions.

CHAPTER 3

MATERIALS AND METHODS

The work flow is indicated as a flow chart in Chapter 1. The materials and methods followed for individual experiments are described below. Some of the common methods are described in appendix.

3.1 Production of Polymer

SCL- MCL polymer (Polyhydroxy propionate co-poly hydroxy ocatadecanoate co-polymer) from *pseudomonas* LDC -5 was produced in our collaborative institute at Madurai, Tamilnadu. Cost effective production of the polymer was standardized by Saranya et al 2010. Solvent casted polymer and its different blends were obtained in powder form as well as thin films.

3.1.1. Cultivation of the bacterium in scale fermenter

- Poly 3- [Hydroxy Propionate (15%) – Hydroxy Octa Decanoate (85%)], a SCL - MCL PHA from *Pseudomonas* sp. MNNG-S was mass produced using 14L scale fermenter shown in **figure 10** (NBS Bio Flo 115, UK) .
- Seed cultures were inoculated into sterilized modified RC medium supplemented with sucrose (10 gL^{-1}, $CaCO_3$ (0.2 $gL^{-1)}$, dihydrogen potassium ortho phosphate (0.5 gL^{-1}) and dipotassium hydrogen phosphate (0.5 gL^{-1}) (36).
- The fermentation parameters included are pH (8), temperature (30 ^{0}C), agitation (350 rpm) and dissolved oxygen (40%).
- The pH was maintained robotically by the addition of 0.5M NaOH and 0.5M HCl. Coconut oil mixed with water (1:1) was used as the antifoaming agent.

3.1.2. Polymer recovery

- Cultured broth was centrifuged at 2000 rpm for 20 minutes at stationary phase.
- PHA was extracted from the cells as per the method described by Sujatha and shenbagarathai, 2005, using sodium hypochlorite and chloroform (75).
- The cell mass was stirred with a dispersion of chloroform (25 ml) and 30% sodium hypochlorite (25 ml) at 37 ^{0}C for 90 minutes.

- It was then centrifuged at 8000 g for 20 minutes at 30 ^{0}C.
- Out of the three phases obtained the bottom chloroform phase containing PHA was recovered by non solvent precipitation using ice cold methanol, 4 to 6 volumes.

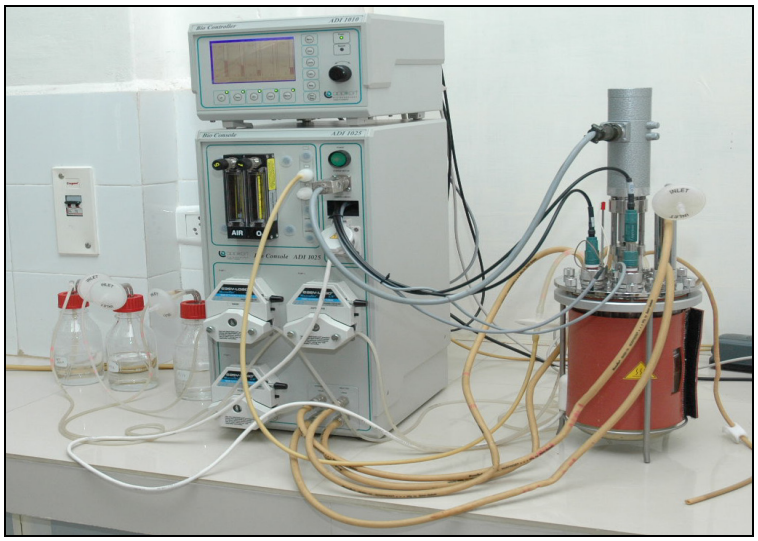


Figure 10:- **A 14L scale fermenter** (NBS Bio Flo 115, UK)

- The recovered white powdery polymer was dissolved in chloroform for further studies.

3.1.3. GC MS (Gas chromatography-Mass spectrometry) analysis for purity of the polymer

- To ascertain purity, a coupled GC-MS was performed using a GC Clarus 500 Perkin Elmer with Elite -1 (100% Dimethyl polysiloxane) capillary column (30 mm X 0.25 mm X ; 1 µm ; carrier gas- He,1ml/min; temperature programme 110 ^{0}C for 2 minutes then increased at 5 ^{0}C/min to 280 ^{0}C and maintained) equipped with a Turbo mass gold model mass selective detector (PPRC, Tanjore).
- The mass spectra obtained were compared with the NIST 98 Mass Spectral Library with windows search program version 2.1, National Institute of Standards and Technology (US department of commerce) with electron energy of 70 eV and mass scan (m/z) 45-50.

3.1.4. Preparation of PHA films (naive and blended) by Solvent casting

- The PHA recovered from *Pseudomonas* sp. MNNG-S was blended with other biodegradable polymers like poly lactic acid (PLA), poly ethylene glycol (PEG)

and montmorillonite (MMT) in the of ratio of 60/40 (w/w) to improve its optical and elastic properties.

- Films of pure PHA and with its blend were prepared by solvent casting method (17).
- The materials were dissolved in chloroform to give 15% (w/v) solutions and were stirred thoroughly at 60 ^{0}C for 10 min.
- Then it was poured onto 14cm Petri dishes and allowed to evaporate in a vacuum dessicator overnight.

3.1.5. Bovine Serum Albumin (BSA) adsorption test

- Protein adsorption onto the surface of PHA films and its blends with PLA, PEG and MMT were estimated to evaluate the capability of scaffolds for cellular interaction.
- The quantum of protein adsorption was determined by BSA adsorption assay. Scaffold films were immersed in 5ml of 0.2% BSA solution for 2h.
- The supernatant solution was carefully collected after incubation to determine the adsorption on the surface of the film using UV spectroscopy (Eppendorf Biophotometer, U.K).
- The absorbance was measured at 280 nm. A calibration curve was prepared using known concentrations of BSA.
- The protein adsorbed (q) on the surface of the film was calculated using the following equation, $q = (c_i\text{-}c_f V)/m$, where Ci is the initial BSA concentration and Cf is the final BSA concentration in the supernatant after adsorption; V is the total volume of the solution (5mL); and m is the weight of the films added into the solution (109).

3.2. Evaluation of the polymer scaffold for continuous cultivation

The polymer scaffold was evaluated for its suitability for continuous culture in terms of viability and proliferation of cultured cells.

3.2.1. Authentication of the continuous cell line HEp-2 Maintained in our laboratory

The authenticity of HEp-2 culture was checked by standardizing DHPLC based STR fingerprinting for cell line. The HEp-2 cells were trypsinized and DNA was extracted (**the protocol is given in appendix)** for amplifying selected STR regions.

3.2.1.1. Polymerase Chain Reaction (PCR)

- The PCR master mix was prepared using components listed in appendix. The primers used are given in **Table 1**.
- For each STR region to be amplified, a test sample and negative control PCR reaction was set.
- HEp-2 DNA extracted previously is added to the test sample at a concentration of 1µg/5µl. DNA was not added to the negative control reaction.
- The vials containing PCR components were then placed in the PCR machine.
- The thermal profile for the PCR reaction was set as given in **Figure 11**.

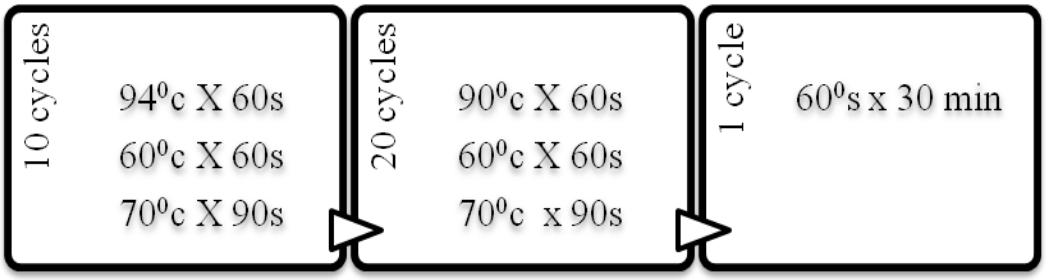


Figure 11:- Thermal Profile for STR amplification. The Figure shows the thermal profile used for amplifying STR regions. The thirty cycle reaction is divided in to 3 parts based on the temperature settings. Each segment has denaturation, annealing and extension step as given above (80).

- Once the thermal cycling was complete, the reaction was held at 4 ^{0}C to cool the products, after which the vials were removed from the PCR machine.
- The success of the PCR was tested by Agarose Gel Electrophoresis of amplicons as given in appendix.

TABLE. 1: Primers Used For Amplifying STR Regions

STR region	Primers
vWA loci	F: 5'-GCCCTAGTGGATGATAAGAATAATCAGTATGTG-3‘ R: 5'- GGACAGATGATAAATACATAGGATGGATGG-3’
DI6S539	F: 5'-GGGGGTCTAAGAGCTTGTAAAAAG-3‘ R: 5'-GTTTGTGTGTGCATCTGTAAGCATGTATC-3'
D7S820	F: 5'-ATGTTGGTCAGGCTGACTATG-3‘ R: 5’-GATTCCACATTTATCCTCATGGA-3’

3.2.1.2. Denaturing high performance liquid chromatography

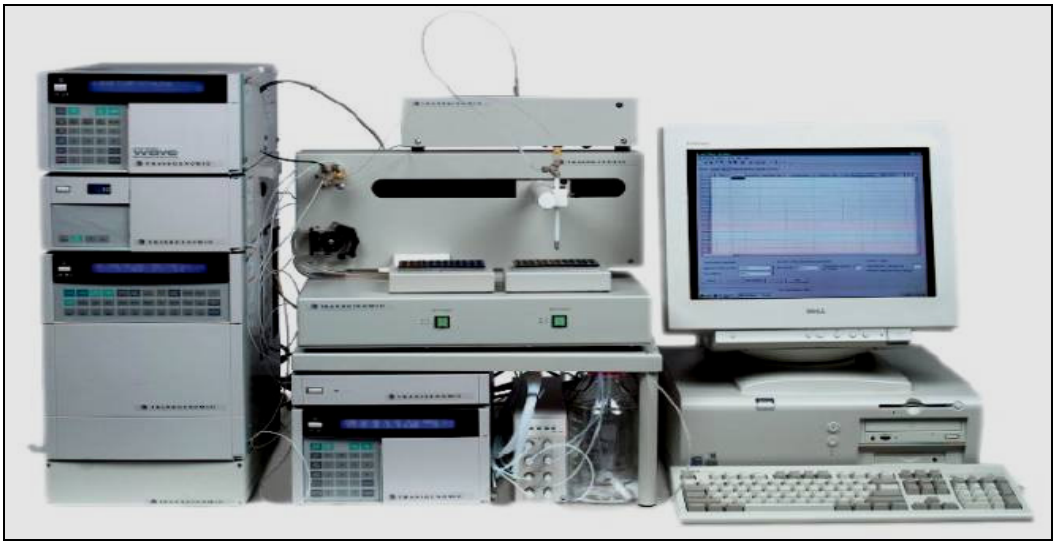


Figure 12:- **Wave DNA Fragment Analysis System**.The system from Transgenomics is exclusively used for separation of nucleic acids.

TABLE. 2: **Materials Required For STR Allele Fragment Separation By DHPLC**

Component	Description
PCR amplicon	8 µl to be injected in the column
DHPLC column	Alkylated poly(styreme-divinylbenzene) particles
Buffer A	0.1M Tetraethylammonium Acetate (TEAA)
Buffer B	0.1M TEAA with 25% Acetonitrile
Buffer D	75% Acetonitrile, 25% water

- Wave DNA Fragment Analysis System (Figure 12) was used for separation of STR alleles from PCR amplicons. The Navigator software was opened to execute the current DHPLC job. The operation windows are shown in **Figure 13**.
- The first step involved creation of a profile for the job. This includes adjusting the temperature, base pair range of the STR to be isolated and buffer flow rate (**Figure 13 A**).
- Based on the above parameters, the DHPLC instrument generates a buffer profile that highlights the time and buffer concentration at which a fragment of particular base pair size is eluted (**Figure 13. B**).
- Once the profile was saved, injections were generated and the job was run until the time estimated (**Figure 13. C**). The result was obtained in the form of peaks which correspond to the STR alleles (**Figure 13. D**).
- The retention time of the peak corresponds to the time of elution of the required STR fragment, as per the profile generated by the software.
- The same PCR product was once again ran as per the specifications cited in the first run, with the addition of a collection profile. The fractions were collected in the Collector,

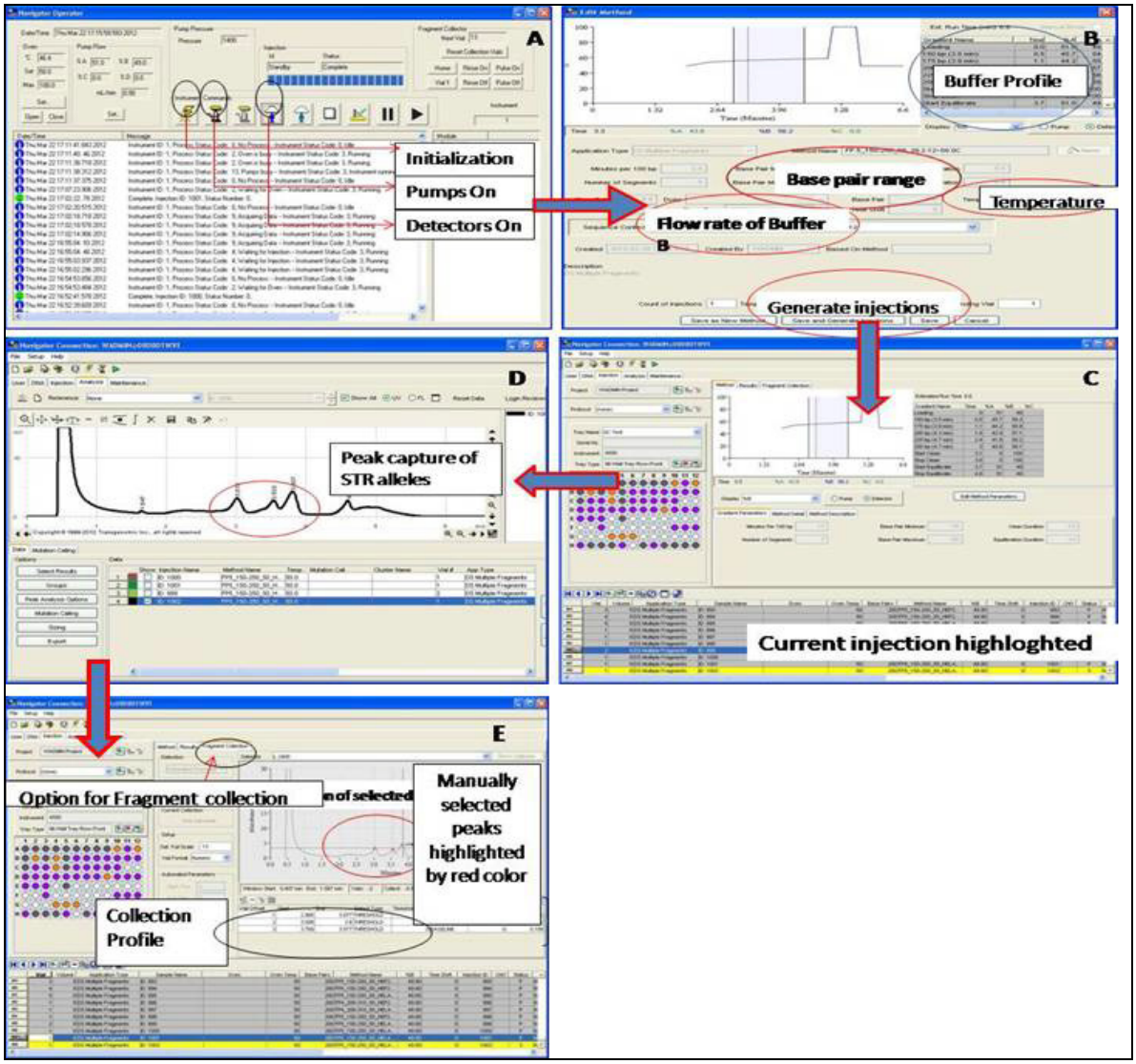


Figure 13:- **Navigator software operations for separation of STR alleles by DHPLC**. The Figure shows the flow of operations from loading of the samples to collection of the fractions; A. Navigator operator window which allows the operator to initialize the program and to turn the detectors and pump on; B. The method window, where, the operator can set up the base pair range, oven temperature and flow rate required; C. Window for injection, which allows the operator to select vial position while loading the sample. Based on the information provided in the method window, the software automatically set up a profile for the run. The selected injection will then be highlighted; D. Analysis window which shows the results in terms of resolution of the peaks for the selected run; E. Fraction collection Window which allows the operator to collect the desired fragments by selecting the corresponding peaks manually, when the same run was setup for the second time to collect the fragment.

when the peaks for the current run, coincided with the peaks selected from the previous run (**Figure 13. F**)

- Once the fractions were obtained, they were concentrated in a SpeedVac concentrator for 2 hours, to ensure that the DHPLC buffers present in the fraction are evaporated.
- The concentrated fractions were resuspended in 30µl of sterile MilliQ water for DNA sequencing.
- The collection of fractions from the instrument requires setting up a new collection profile (**Figure 10**). This was done by manually selecting the peaks obtained from the first run.

3.2.1.3. DNA sequencing

The PCR amplicons were cycle sequenced and purified prior to sequencing as described in appendix. The sequence of the PCR amplified DNA was deduced with the help of ABI Prism 3130 AVANT (Applied Biosystems, USA) genetic analyzer which works on the principle of Sanger's dideoxy sequencing method. The amplified products with the dye at the terminated 3'-end was subjected to Capillary Electrophoresis by an automated sample injection. The emitted fluorescence from the dye labels on crossing the laser area, was collected at the rate of one nucleotide per second by Cooled, Charge-coupled Device (CCD) at particular wavelength bands (virtual fillers) and was stored as digital signals on the computer for processing and the output was read by the machine.

3.2.2. Sterilization or disinfection of the Polymer

As the PHA scaffold is heat sensitive, the method of sterilization or disinfection was validated by a comparative study of polymer sterilized with Ethylene oxide sterilization (EtO sterilization) and disinfected with graded ethanol. The protocols for both the methods are given in annexures.

3.2.3. Validation of the effect of sterilizing agent on cell viability using HEp-2 and Vero cells

3.2.3.1: Seeding of HEp-2 and Vero cells onto the polymer:

- ETO and ethanol sterilized polymer scaffolds were placed in 96 well plates and conditioned with sterile DMEM / F12.

- These conditioned scaffolds were kept for sterility check at 37 ^{0}C CO_2 incubator.
- One flask each of both the cell lines were trypsinized using the protocol given in - appendix
- The trypsinized cells were counted using Neubauers counting chamber by trypan blue exclusion method.
- Fifty microliter of cell suspension containing 5,000 cells was seeded carefully on to the scaffolds as a single drop.
- Cells were also seeded on to 96 well plate surfaces as control.
- The seeded cells were incubated at 37 ^{0}C, 5 % CO_2 incubator.
- MTT assay for cytotoxicity was done on the third day after seeding of cells.

3.2.3.2. Counting of cells by trypan blue exclusion method

- Trypan blue exclusion is a dye exclusion method where only the membrane of the live cells excludes the trypan blue and the cell remains unstained. Dead cell membrane is permeable to the dye and stains cells blue.
- 10 µl of the cell suspension was taken and mixed with 10 µl of the 0.4 % trypan blue in PBS.
- The chamber was loaded with 10 µl of the mixture.
- The total number of dead and live cells was counted separately to calculate the % viability as described in appendix.

3.2.3.3. MTT Assay

MTT assay measures the cell viability and proliferation and forms the basis of numerous *in vitro* assays of a cell population's response to external factors. MTT [3- (4,5-dimethyl thiazol-2-yl)-2,5,-diphenyl tetrazolium bromide] assay first described by Mosmann in 1983, is based on the mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium of the pale yellow MTT and form a dark blue formazan crystals which are largely impermeable to cell membranes, thus results in its accumulation in healthy cells. Solublization of the cells by the addition of a detergent results in the liberation of crystals

which are solubilized. The number of surviving cells is directly proportional to the level of formazan product produced. The colour can then be quantified using simple colorimetric assay. The results can be read on a multiwell scanning spectrophotometer (Biotek Powerwave XS) (MOSMAN T rapid colorimetric assay (110).

Protocol for MTT assay

- After 24 hours of incubation of the cells, the media was removed.
- Fresh media (100 µl) which contained 20 µl MTT (5mg/ml) was added and incubated for 4 hours.
- The crystals were dissolved by adding 150 µl of DMSO
- This was read at 570nm.

3.3. Assessment of biocompatibility of PHA scaffolds with continuous culture of HeLa, HEp-2, Vero and McCoy cells

3.3.1 Cell viability assay

- To evaluate the bio compatibility of the film (pure PHA and PHA blends with PEG, PLA and MMT) with respect to the continuous cell cultures, the uniformly cut and weighed films were placed in a 96 well plate in triplicates.
- 50 µl of DMEM (from Gibco) was added and the ethanol sterilized matrices were kept overnight at 37 ^{0}C for sterility check.
- After sterility check the media was removed and cell suspensions of continuous cultures HeLa (NCCS Pune), HEp-2 (NCCS Pune), Vero (NCCS Pune), McCoy (NCCS Pune) were inoculated (5000 cells per 100 µl DMEM with 10% FBS) onto the sterilized matrix film.
- Similarly cells were inoculated onto the tissue culture plate as control.
- After overnight incubation the medium was replaced by serum free DMEM. 10 µl 5mg/ml MTT (sigma) in PBS was added and incubated at room temperature for 4hours.
- The purple formazan crystals were dissolved by adding 150 µl DMSO (Sigma)

- Reading was taken at 450 nm using ELISA 96 well plate reader (PowerWave HT Microplate Spectrophotometer).

3.3.2. Cell proliferation assay with HeLa, HEp-2, Vero and McCoy cell lines

- To evaluate the proliferation rate of continuous cells on the matrix, the cells proliferating on matrix as well as controls were estimated using colorimetric cell proliferation ELISA, BrdU, kit (Roche Applied Science) after 24 hrs of cultivation.
- Reading was taken at 370 nm using ELISA 96 well plate reader (PowerWave HT Microplate Spectrophotometer).

3.3.3. Evaluation of Cellular Morphology

3.3.3.1. Phase contrast microscopy

Continuous cell lines, HeLa, HEp-2, Vero, and McCoy were seeded onto PHA: PEG polymer. Morphology was evaluated by Phase Contrast Microscopy (Carl Ziess Axio Observer Microscope).

3.3.3.2. Scanning electron microscopy

- HeLa and McCoy cells on PHA: PEG matrix was immersed in 3% glutaraldehyde buffered with 0.1 M phosphate buffer at room temperature for 2-4 h.
- The samples were then washed thrice in 0.1 M phosphate buffer (pH-7.2).
- The samples were then immersed in 1-2% osmium tetroxide solution and washed thrice.
- Dehydration was typically done by passing the specimens through a graded series of ethanol-water mixtures – 30%, 50%, 70%, 80%, 90%, 96%, and 100% for 5-15 min each and then dried. The cells grown over matrix were documented by Scanning Electron Microscopy.

3.4. Evaluation of the polymer for limbal cultivation

3.4.1. Preparation of limbal tissues

- Transplant rejected corneal rim or whole globes were transported in DMEM /F12 medium from CU Shah eye bank.

- Corneal rims or whole globes were washed in 1% antibiotic solution containing DMEM /F12.
- Limbal biopsies were taken carefully from the cornea-conjunctival junction by using corneal scissors and forceps.
- Alternatively tiny bits of corneal limbal tissues were collected from the donor corneas during the preparation of corneal rim for transplantation.

3.4.2. Corneal limbal explant culture

- Ethanol sterilized PHA: PEG scaffolds were cut uniformly and placed in 6 well plates.
- Scaffolds were then conditioned in DMEM /F12 with 10% FBS and antibiotics (100 U/ml penicillin and 100 pg/ml streptomycin)
- Tiny limbal biopsies were placed over the scaffold
- A drop of DMEM/F12 with 10% FBS and antibiotics (100 U/ml penicillin and 100 pg/ml streptomycin) was left over the tissue bit in order to prevent drying.
- After overnight incubation scaffold was covered with enough medium.

3.4.3. Evaluation of outgrowth

Outgrowth of limbal explants over the matrix was observed every day under phase contrast microscopy.

3.4.4. Histopathological sectioning

A scaffold which had a confluent growth of corneal limbal epithelial cells subjected to histopathological sectioning with the help of Department of Pathology, Sankara Nethralaya, to examine the degree of differentiation and stratification. The brief procedure followed is as given below.

- The scaffold with the cells was fixed with neutral buffered formalin for 48 hours at room temperature
- After fixation of the cells the scaffold was processed for dehydration with graded ethanol baths (40%,70%, 90% and absolute ethanol)

- Then the sections were subjected to clearing with two changes of xylene each for 1 hr.
- The scaffold was then embedded with paraffin wax at 58 ^{0}C
- The embedded scaffolds were placed in a cassette with molten paraffin to form a paraffin block.
- This was then subjected for vertical sectioning using microtome.
- The sections were carefully picked up with silane coated slides after the sections were allowed to float in tissue floatation water bath.
- The slides were then processed for Hematoxylin and Eosin (H& E) staining.

3.4.5. H & E staining

- The sections were deparaffinized with 2 changes of xylene 10 minute each.
- Then it was rehydrated in 2 changes of absolute alcohol, 5 minutes each, 95% alcohol for 2 minutes and 70% alcohol for 2 minutes.
- The slide was then washed with distilled water briefly followed by staining in Harris Hematoxylin solution for 8 minutes.
- Then the sections were washed in running tap water for 5 minutes.
- These sections were then differentiated in 1% acid alcohol for 30 seconds followed by wash in running tap water for 1 minute.
- Bluing of the sections was done with 0.2% ammonia water for 1 minute followed by washing in running tap water for 5 minutes.
- The counterstaining was done with Eosin – Phloxin solution for 30 seconds.
- The sections were then dehydrated with grade ethanol, cleared with xylene and mounted with DPX.
- The sections were observed under light microscope

3.4.6. Fluorescent staining of the nuclei by DAPI

- In order to confirm the stratification of corneal limbal epithelium, vertical sections of the scaffold was subjected to DAPI staining for nuclei.
- The paraffin sections were de waxed in xylene (2 x 5min) and rehydrated in ethanol series (absolute, 95% for 5 min, 70%, 30% ethanol, dH2O for 3 min),
- Equilibrated the slides in PBS (5 min, prepared fresh).
- Drained the slides and put them on paper towel.
- DAPI staining solution was dropped on to the slide and incubated for 15 min in dark.
- The excess solution was drained and mounted with glycerin.
- Observed with UV excitation at Carl Zeiss Axio observer microscope.

3.4.7. Rate of corneal limbal epithelial cell proliferation on PHA: PEG scaffold compared to control tissue culture surface

- To evaluate the proliferation rate of primary corneal limbal epithelial cells on the matrix, the tissue biopsies were placed in both PHA: PEG matrix and cell culture plate.
- Cell proliferation on matrix as well as controls were estimated using colorimetric cell proliferation ELISA, BrdU, kit (Roche Applied Science) on day-1, day-2 and day-3.
- Reading was taken at 370 nm using ELISA 96 well plate reader (Power Wave HT Microplate Spectrophotometer).

3.4.8. Qualitative RT PCR For the expression of markers

RNA was extracted from the cultures on Day 2 and Day 12 using Qiagen RNeasy minikit. Qualitative RT-PCR was done for the limbal stem cell markers, *ABCG-2*, *Np 63* and corneal epithelial differentiated markers *K3* and *K12* along with *GAPDH*. Primers used, anneling temperature and product size are listed in the **Table 3**.

3.5. Evaluation of the scope of the polymer to support a 3d HSV-1 culture

3.5.1. Comparative evaluation of the morphology of cells grown in various matrices

To evaluate the scope of the polymer to support a 3d HSV-1 culture the commercially available 3d culture systems, Hydromax peptide cell culture scaffold, matrigel, mebiol gel, Polyhydroxy alkanoate, were compared in terms of morphology and proliferation

TABLE 3: List of Primers Used For Expression Study

Marker	Forward Primer (5'- 3')	Reverse Primer (5'- 3')	Base pair size
ΔNp63	CAGACTCAATTTAGTGAAG	AGCTCATGGTTGGGGCAC	440
ABCG-2	AGTTCCATGGCACTGGCCATA	TCAGGTAGGCAATTGTGAAGG	379
Connexin	CCTTCTTGCTGATCCAGTGGTAC	ACCAAGGACACCACCAGCAT	154
K3	GGCAGAGATCGAGGGTCTC	GTCATCCTTCGCCTGCTGTAG	145
K12	CATGAAGAAGAACCACGAGGATG	TCTGCTCAGCGATGGTTTCA	150
GAPDH	GCCAAGGTCATCCATGACAAC	GTCCACCACCCTGTTGCTGTA	498

3.5.1.1. Preparation of matrices (Hydromax peptide cell culture scaffold, matrigel, mebiol gel, Polyhydroxy alkanoate) for cell culture

The commercially available polymer gels were processed as per the manufacturer's instruction. The processing of each polymer before cell culture is detailed below.

3.5.1.1.1. Preparation of hydromatrix peptide nanofiber scaffold

HydroMatrix is a peptide nanofiber 3-dimensional scaffold, obtained from Sigma, which promotes cell growth and migration. HydroMatrix utilizes specific peptides that self assemble from fluid precursors into highly cross-linked peptide hydrogels in response to increases in temperature or ionic strength. Induction of the rapid solution-gel transformation of this peptide solution generates a peptide nanofiber scaffold.

- The product was supplied as a lyophilized powder.
- A 1% (w/v) stock solution (10 mg/ml) was prepared by addition of cold sterile water.
- Since 1% (w/v) stock solution resulted in a rigid gel a 0.5% (w/v) working solution was prepared which resulted in a softer gel.
- Seventy five micro liters of working solution of the gel was added carefully onto the 96 well microplate after spinning down to remove the air bubbles formed.
- Since the formation of gel depends on pH, to enhance the gel formation 1 volume of the cold medium (DMEM /F12) was added to the side of each well without disturbing the gel solution and kept at 37 ^{0}C for 1 hour to allow the gel to form.
- Then carefully changed the medium twice at the interval of 1-2 hours.
- Gelled plate was kept at 37 ^{0}C till the cells were ready (but not more than 5 hours).

3.5.1.1.2. Preparation of Matrigel

- BD Matrigel™ Basement Membrane Matrix Growth Factor Reduced was diluted as recommended by the manufacturer.
- Using cooled pipets, the BD Matrigel Matrix GFR was mixed to homogeneity.

- Added 50 µl of the diluted BD Matrigel Matrix GFR to 96 well plates being coated.
- It was then incubated at room temperature for one hour. Unbound material was then aspirated and rinsed gently using serum-free medium.

3.5.1.1.3. **Preparation of Mebiol gel**

- Mebiol gel was obtained from Nichi-In Centre for Regenerative Medicine (NCRM).
- Ten ml of DMEM /F12 was added on to the bottle of gel to reconstitute and kept at 4 ^{0}C for 24 hours or till the gel liquefied fully.
- The Mebiol gel was then plated (50 µl) on to the 96 well plates, keeping both the plate and liquefied gel on ice.
- The coated plate was then kept at 37 ^{0}C until the gel was formed.

3.5.1.2. Seeding of Human Corneal Epithelial Cells (HCE) on the Scaffolds

- After overnight incubation of the PHA: PEG in DMEM /F12 and formation of the other scaffold gels, the cells were trypsinized.
- Fifty microlitre suspension of HCE cells containing 10,000 cells were seeded on to each scaffold.
- The cells were carefully added without spilling out.
- Equal amount of cells were seeded onto the surface of the 96 well plates as control 2d culture.

3.5.2. Evaluation of Cellular Morphology

Cellular morphology of each system was analyzed after overnight incubation.

3.5.3. Evaluation of Cellular Proliferation

The cell proliferation rate in each system was analyzed using BrdU ELISA kit after 48 hours of culture.

3.5.4. Comparative evaluation of viral multiplication and immune status of cells when exposed to HSV-1 infection

Rate of viral multiplication and status of immune response of the cells in response to infection was analyzed after infecting the cell culture system with 0.5 multiplicity of infection (MOI).

3.5.4.1. Evaluation of titer of the stock virus

Vero culture supernatant of HSV-1 standard strain (KOS), frozen at -80^{0}C, was thawed and titer of the stock virus was determined by plaque assay. Protocol for the plaque assay is as follows.

3.5.4.1.1. Plaque assay protocol

3.5.4.1.1.1. Cultivation of cells

- HCE cells were trypsinized and 2ml (1 x 10^6 cells /ml) of growth media with cells were added on to each well of a 6 well plate.
- The plates were incubated in a 37 ^{0}C, CO_2 incubator for 12-24 hours.

3.5.4.1.1.2. Preparation of viral dilution

- Prepared 4 tubes containing 2 ml of PBS.
- Twenty micro litre of virus sample was added to the first tube and vortex mixed.
- The dilution process was repeated through all the four tubes to obtain a final effective dilutions of virus 10^{-2}(1/100), 10^{-4} (1/10,000), 10^{-6} (1/1,000,000), 10^{-8} (1/100,000,000).

3.5.4.1.1.3. Infection of the cell culture

- One ml of media was discarded from each well leaving one ml in each well.
- Added 100 µl of each dilution in duplicate to each well, letting the virus flow gently into the media.
- The infected monolayers were incubated at 37 ^{0}C for four hours after keeping the plates 15 minute in shaker to enhance adsorption of the virus.

3.5.4.1.1.4. Agar Overlay

- A sterile solution of 4% agarose in dH_2O was prepared by autoclaving at 121 ^{0}C for 20 minutes.
- The plaquing media was incubated in a 37 ^{0}C water bath until equilibrated.
- The media was gently drawn out of each HCE infected monolayer well and discarded.
- The volume of media needed was kept in a 37 ^{0}C pre-warmed container and added 0.11 volumes of liquid agarose to the bottle with swirling (1:10 dilution) with vigorous shaking to mix.
- Two ml of the agarose/growth media mixture was added to each well immediately but gently.
- The plate was incubated for 15 minutes in the level hood at room temperature till the agar overlay turned solid. The plates were then moved to a humidified incubator at 37 ^{0}C and 7.5 to 10% CO_2.

3.5.4.1.1.5. Plaque visualization

- Plaques were visualized by staining after 3 days of infection by staining the monolayer by MTT 0.1 volume of MTT solution (5 mg/ml in PBS) (Sigma, M2128).
- The plaques were counted with naked eye.
- After counting plaques, the concentration of the initial viral suspension was calculated in PFU/ml or multiplicity of infection (MOI).

3.5.4.1.2. Infection of HCE in Different Polymers by Diluted Virus

- The stock virus after determining the titer was diluted to obtain 0.5 MOI.

  HCE in each polymer system was infected with 20 µl diluted stock viral supernatant.

3.5.4.1.3. Evaluation of morphology of HCE cells before and after infection

The cells in each system were evaluated by phase contrast microscopy, before and after infection, till a visible cytopathic effect (CPE) for HSV was noticed in control 2d system.

3.5.4.2. Evaluation of multiplication of virus in each system before and after infection

- After 48 hours of incubation the supernatant was collected from each system.
- Total DNA was extracted as described in appendix.
- HSV-1 real time assay was performed using Gene Sen's HSV -1 real time assay kit.
- Prepared master mix following manufacturer's instruction as given in the **Table 4**.

TABLE. 4: Preparation of Master Mix For HSV-1 Real Time PCR

Number of reactions	For One Reaction	For Ten Reactions
HSV-1 Super mix (R1)	12	120
HSV-1 mg solution (R2)	3	30
Total	15	150

- Pipetted 15 µl of the Master Mix into each labeled PCR tube
- Then 10 µl of the earlier extracted DNA was added to each sample tube and mixed well by pipetting up and down.
- Correspondingly, 10 µl of the Standards (HSV 1 -S1-5) was used as a positive control and 10 µl of water (Water, PCR grade) was used as a negative control.
- The PCR tubes were closed and transferred into the rotor of the Rotor Gene™ instrument.

3.5.4.3. Evaluation of TLR expression of the cells with and without HSV -1 infection

Total RNA was extracted from treated and control cells at 24 hours of infection using RNA extraction kit from Qiagen (given in appendix). First strand complementary DNA (cDNA) was synthesized from 1µg of total RNA using oligo dT primers (Fermentas, USA) and sensiscript RT (Qiagen, Hilden, Germany) as per the manufacture's protocol (given in appendix). .

- Real time PCR was performed for 18S, TLR 1, TLR 2, TLR 6 and TLR 9 and the reaction was amplified by quantiTect SYBR green master mix (Qiagen, Hilden, Germany) on ABI-7300 machine.
- Relative gene expression levels were calculated by normalizing with corresponding 18S transcript levels and expressed as relative fold change compared with 2d control.

CHAPTER 4

RESULTS

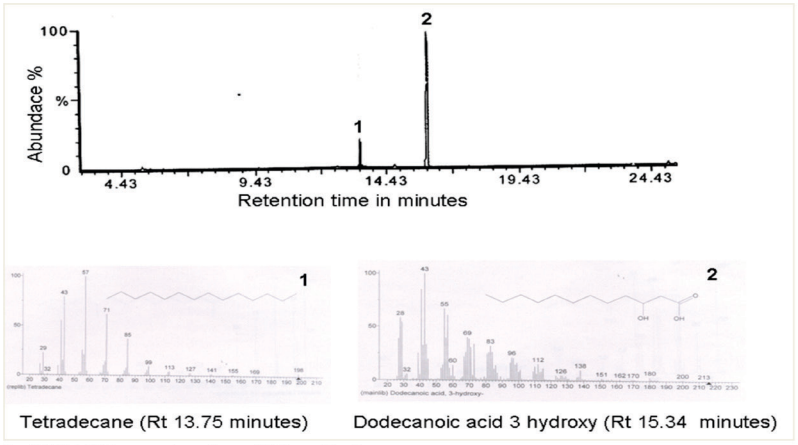
The study was aimed at validating the scope of indigenously produced PHA from *Pseudomonas MNNG-S,* based copolymer in continuous as well as primary corneal limbal epithelial cultures. The study went further in evaluating the potential of the polymer to support a 3d culture system, as it was found suitable for continuous culture, for viral infectivity studies. The polymer was produced using 14 L scale fermenter. After checking the purity of the polymer, it was subjected for solvent casting. Blending studies with other biodegradable polymers were done in order to improve the polymer properties to make the polymer more suitable for cell culture. The stock HEp-2 culture maintained in our laboratory was subjected to cell line authentication by standardizing a DHPLC based STR profiling system before proceeding to the cell culture experiments. The individual results of the entire study are discussed below.

4.1 Production, purity assessment and scaffolding of the Polymer PHA

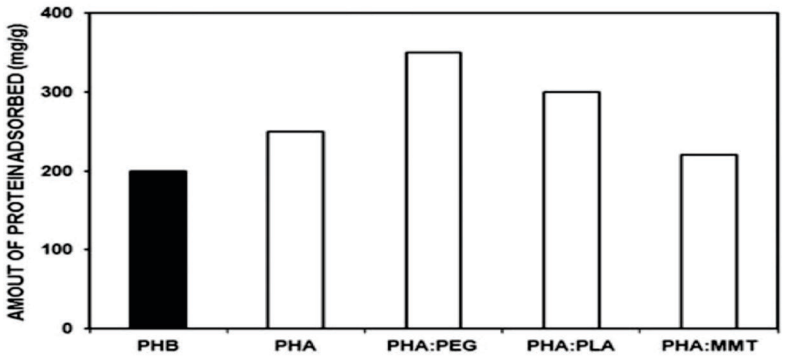
Scaffolds in 3d architecture were prepared after the purity check by GC MS analysis, where in two peaks were resolved; One at 14.43 corresponding to SCL polymer and the other at 15.34 corresponding to MCL **(Figure 14. I)**. Thus it is confirmed that the polymer produced was a pure mixture of PHA SCL and MCL. The pore diameter of the solvent casted scaffold was around 40 μm as measured by Scanning Electron Microscopy (Figure 14 - II). The pore diameter makes it suitable for the growth of cellular constructs. Out of the different blends produced, PHA: PLA **(Figure 14. II A)** matrices were hygroscopic while PHA: MMT matrices **(Figure 14 - II B)** were highly amorphous. PHA: PEG matrices **(Figure 14. II C)** were transparent, clear and in a sheet like elastic form.

4.1.3 Protein adsorption studies

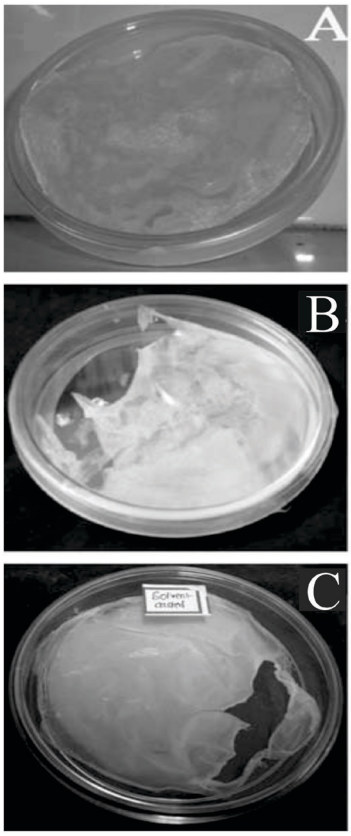
The attachment of cells onto the matrices is mediated by adsorbed proteins and the underlying surface chemistry controls protein adsorption. **Figure 14. III** indicates that PHA: PEG blended film had adsorbed higher amount of protein (340 mg/g) compared to PHA: PLA (317 mg/g). Other films (Naïve PHA, PHB standard and PHA: MMT) had adsorbed relatively lesser proteins. Minimal protein adsorption was noted in PHB standard (210 mg/g).



I: GC MS analysis of the Polymer



III: Protein adsorption chart of different PHA blends



II: Solvent Casted PHA and it's blends

Figure 14:- Purity ascertainment, processing of the polymer PHA and blending studies. I- GC MS analysis of the polymer. The chromatogram shows 2 peaks; one at a retention time of 13.75 corresponding to SCL and the other at 15.34 corresponding to MCL; II- Preparation of PHA based blends by solvent casting method. A, B & C- Solvent casted films of PHA: PLA, PHA: MMT and PHA: PEG, respectively. III- Chart showing the results of protein adsorption studies, the amount of protein adsorbed onto each blends.

4.2. Evaluation of the polymer scaffold for continuous cultivation

The HEp-2 culture was authenticated before performing the evaluation of polymer scaffold for continuous cultures

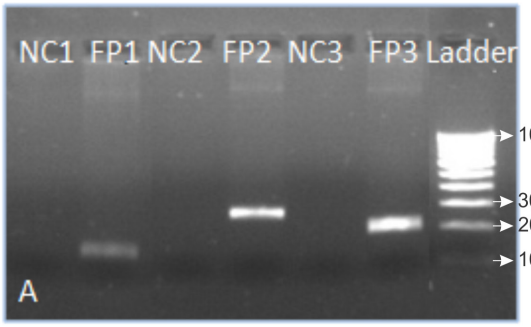
4.2.1. Authentication of the continuous cell line HEp-2 maintained in our laboratory, using DHPLC based STR fingerprinting

STR profiling was standardized using DHPLC for authentication of HEp-2. For this STR locus was amplified using specific primers. Allelic fractions were separated from each amplicons by standardizing DHPLC profile. The fractions were DNA sequenced, in order

to validate the standardized profile and to count the number of repeats in each fraction manually.

4.2.1. Amplification of STR regions by Polymerase Chain Reaction

Figure 15 shows the electrophoretogram of the amplified STR regions. The base pair range of each locus is listed in the Table.



NC1 – Negative Control for Primer 1
FP1 – Fingerprinting Primer 1
NC2 – Negative Control for Primer 2
FP2 – Fingerprinting Primer 2
NC3 – Negative Control for Primer 3
FP3 – Fingerprinting Primer 3

Primer	STR Region amplified	Base Pair Range (bp)
FP1	vWA	127-167
FP2	D16S539	264-304
FP3	D7S820	215-247

Figure 15:- Results of PCR for the three STR loci. A- Electrophoretogram of the STR locus amplified. The table shows base pair range for each PCR product**.**

4.2.2. Standardization of DHPLC based STR finger printing using 3STR loci

The three loci selected for standardization were vWA, D16S539, D7S820. Standardization of each locus is divided into two parts. In part I, the profile was standardized by trial and error method using different oven temperatures and buffer profiles based on the base pair range of each STR locus. In part II, after getting well separated allelic fractions, the separation was validated and number of repeat unit was counted using the DNA sequencing technique.

4.2.2.1. Standardization of allelic separation of D7S820 locus using DHPLC

The D7S820 locus is 215-247 bp in size. The PCR product containing amplified D7S820 STR is run in 2 different profiles, by varying the base pair range for the runs. The flow rate is maintained as 0.9 ml/min.

1. Buffer profile for 100-300 base pair range

- Temperature: 50 ^{0}C, 58 ^{0}C, 60 ^{0}C (Non-denaturing, partially denaturing and fully denaturing conditions)

2. Buffer profile for 150-250 base pair range

- Temperature: 50 ^{0}C

As given in Figure 16, well resolved peaks were not observed at 60 ^{0}C and 58 ^{0}C, indicating that STR alleles are not separated. Peak resolution was good at 50 ^{0}C, but it revealed 3 peaks against the two expected. So another profile (profile 2) was set with a lesser base pair range in order to enhance specificity. However, three well resolved peaks were observed at profile 2 also as shown in **Figure 17** Separation of peak one and peak two were comparatively good at profile 2 and hence all the three fractions were collected by using the buffer profile 2, for sequencing.

4.2.2.2. DNA Sequencing data allelic fractions collected for D7S820 locus:

The allelic fractions of D7S820 locus were collected from DHPLC using the resolved peaks corresponding to the profile 2 at 50 ^{0}C. **Figure 18** shows the sequence data. Considering the sequencing data, peak 2 seemed to be similar to peak 1 with a sequence variation only at flanking region and not at the STR region as the number of repeat units being the same in both the fractions. Both the fractions have 8 repeats of the sequence ATAG. Fraction 3 revealed 12 repeats of ATAG.

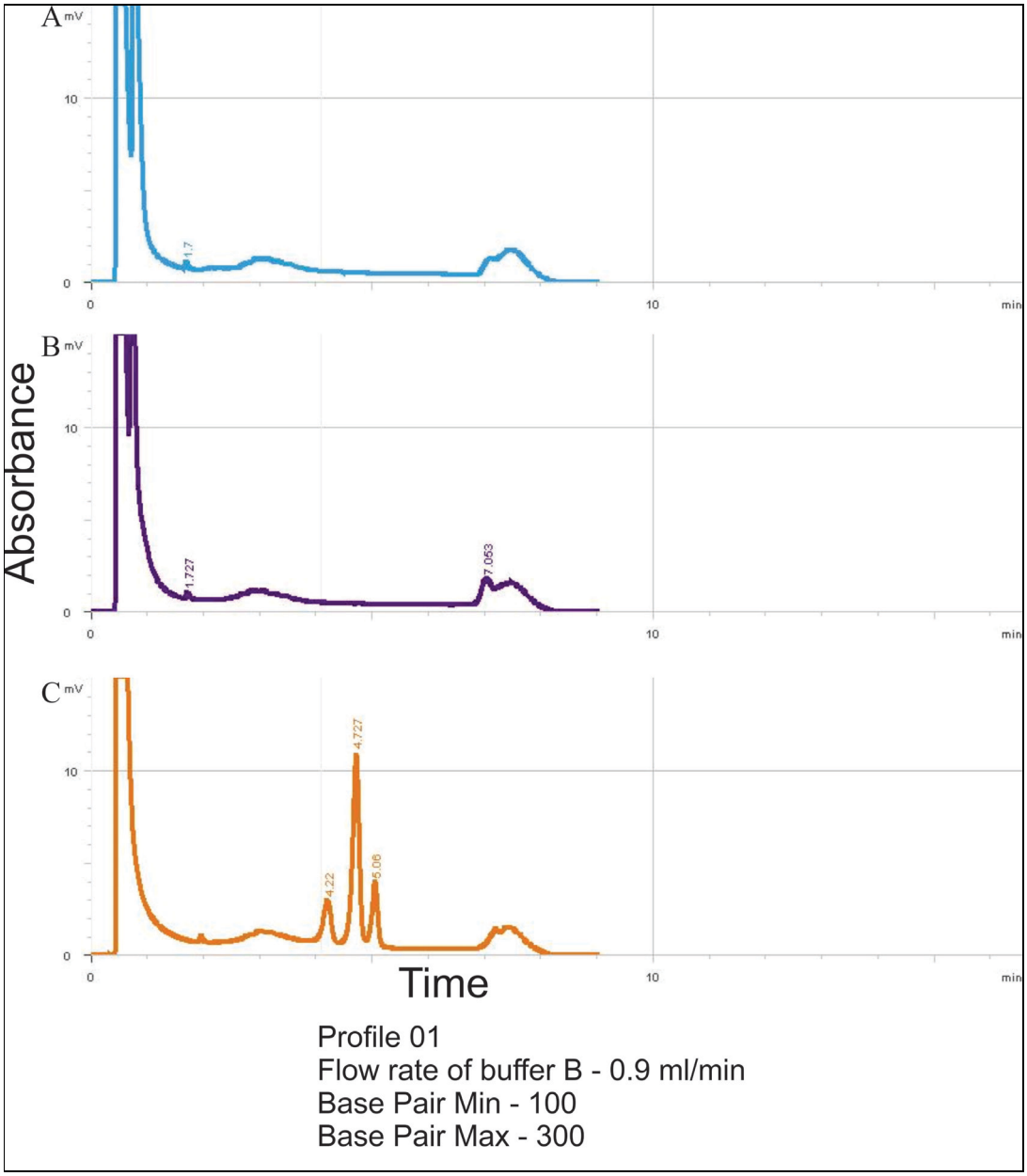


Figure 16:- Standardization of D7S820 locus using buffer profile 1. A- DHPLC chromatogram at 60 ^{0}C; B- DHPLC chromatogram at 58 ^{0}C; C- DHPLC chromatogram at 50 ^{0}C. Flow rate, base pair minimum and bas pair maximum of profile 1 are given below the chromatogram.

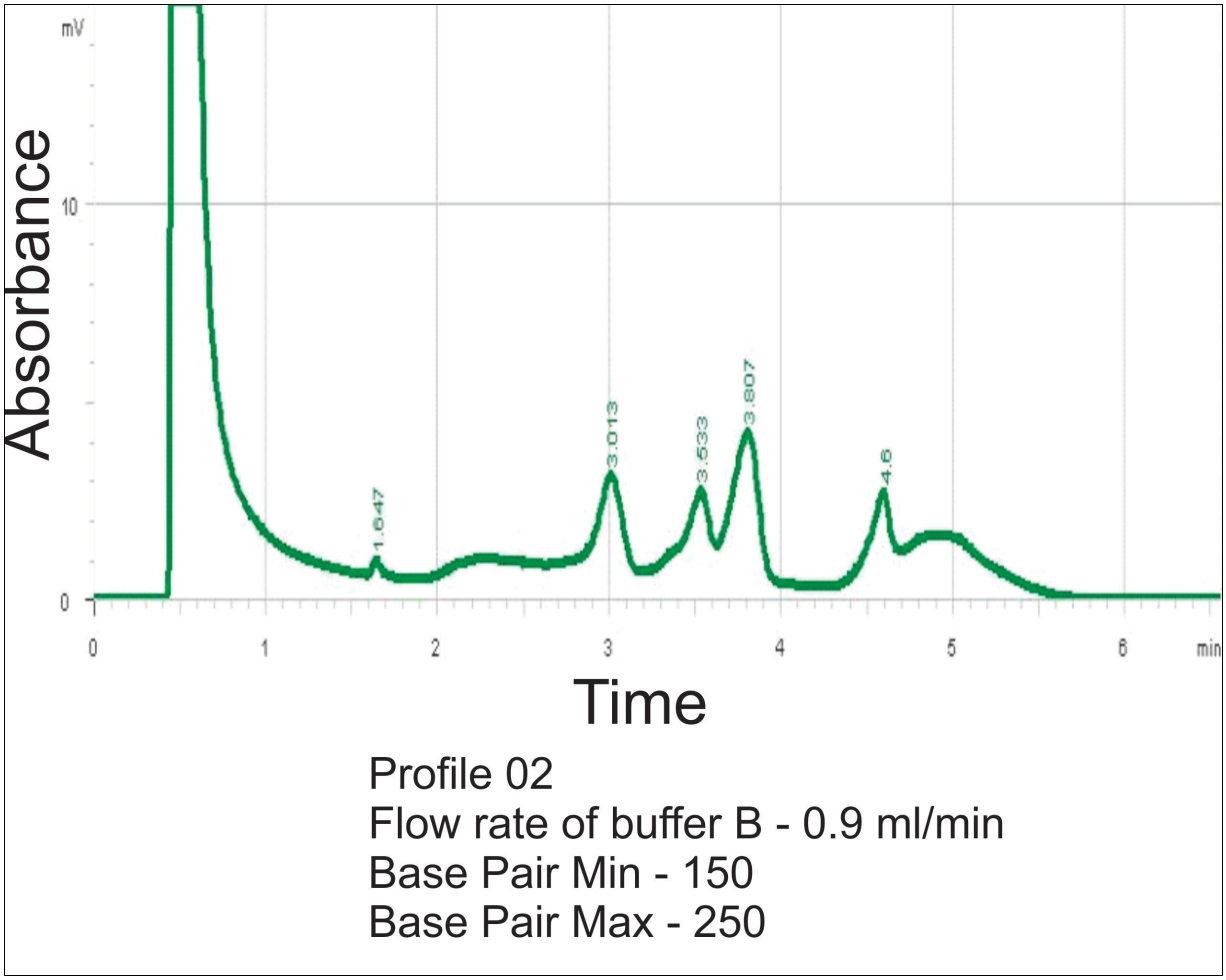


Figure 17:- Standardization of D7S820 locus using buffer profile 2. The Figure shows DHPLC chromatogram at 50 ^{0} C. Flow rate, base pair minimum and bas pair maximum of profile 2 are given below the chromatogram.. Well resolved peaks were obtained at 50 ^{0}C**.**

Thus it is concluded that the isolation of D7S820 alleles from PCR product using DHPLC was more efficient at an oven temperature of 50 ^{0}C, regardless of minor changes in base pair range and buffer profiles used. Selection of lower base pair range resulted in extra peaks which had minor sequence variation at the flanking region.

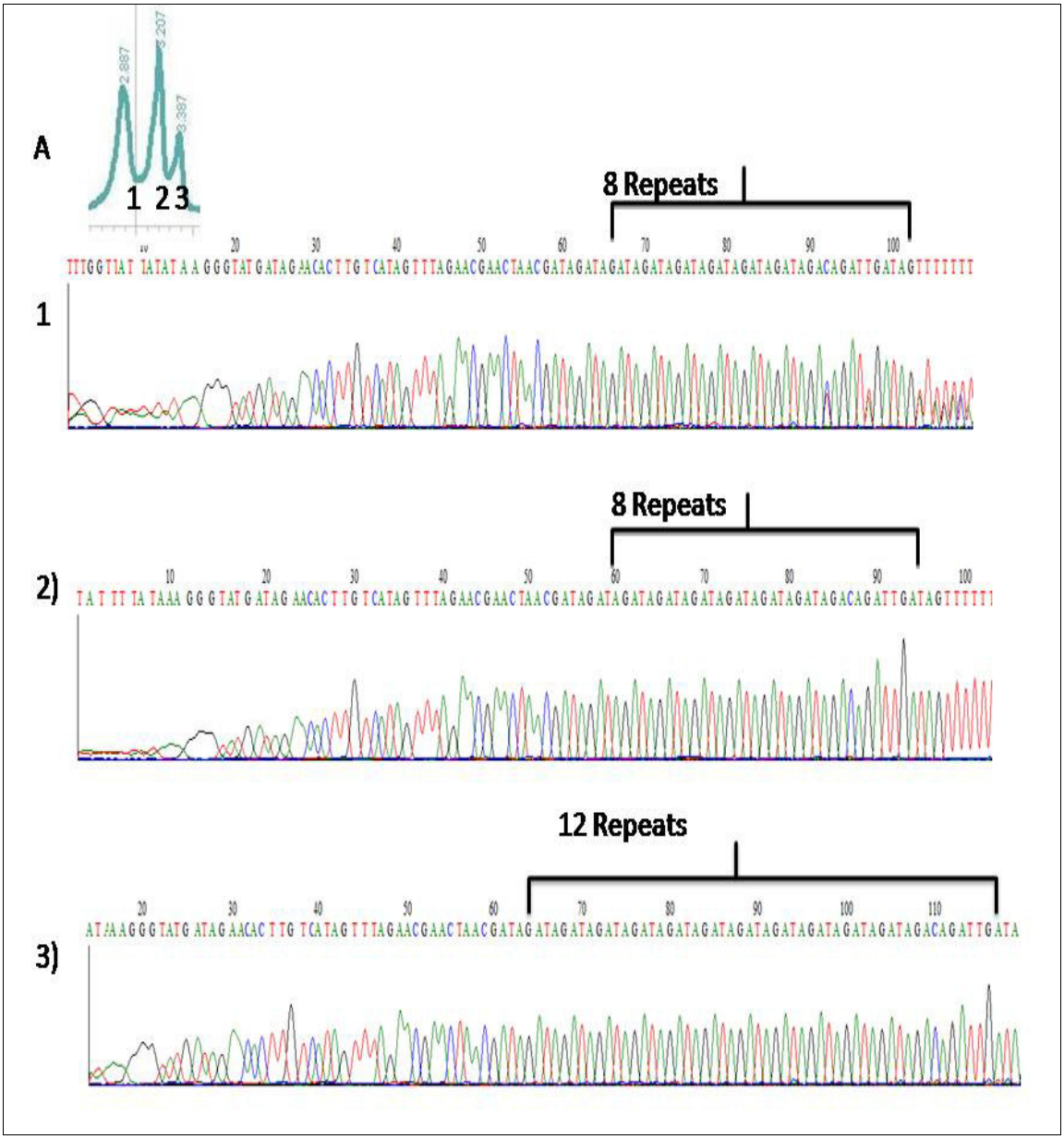


Figure 18:- DNA sequence data of allelic fractions collected for D7S820 locus. A. Peaks corresponding to fractions collected numbered 1, 2 and 3. The corresponding sequences obtained by DNA sequencing of the fractions were numbered accordingly. The fractions 1 & 2 corresponding to peaks 1 & 2, showed 8 repeats and fraction 3 corresponding to peak 3 showed 12 repeats.

4.2.2.3 Standardization of allelic separation of vWA locus using DHPLC

The vWA loci is about 127-167 bp in size. The base pair range for this locus was entered as 100-200 in the DHPLC for generating buffer profile corresponding to this range. The temperatures used were 50 ^{0}C for non-denaturing conditions and 58^{0}C for partially denaturing conditions. The flow rate of buffer B was maintained at 0.9ml/min.

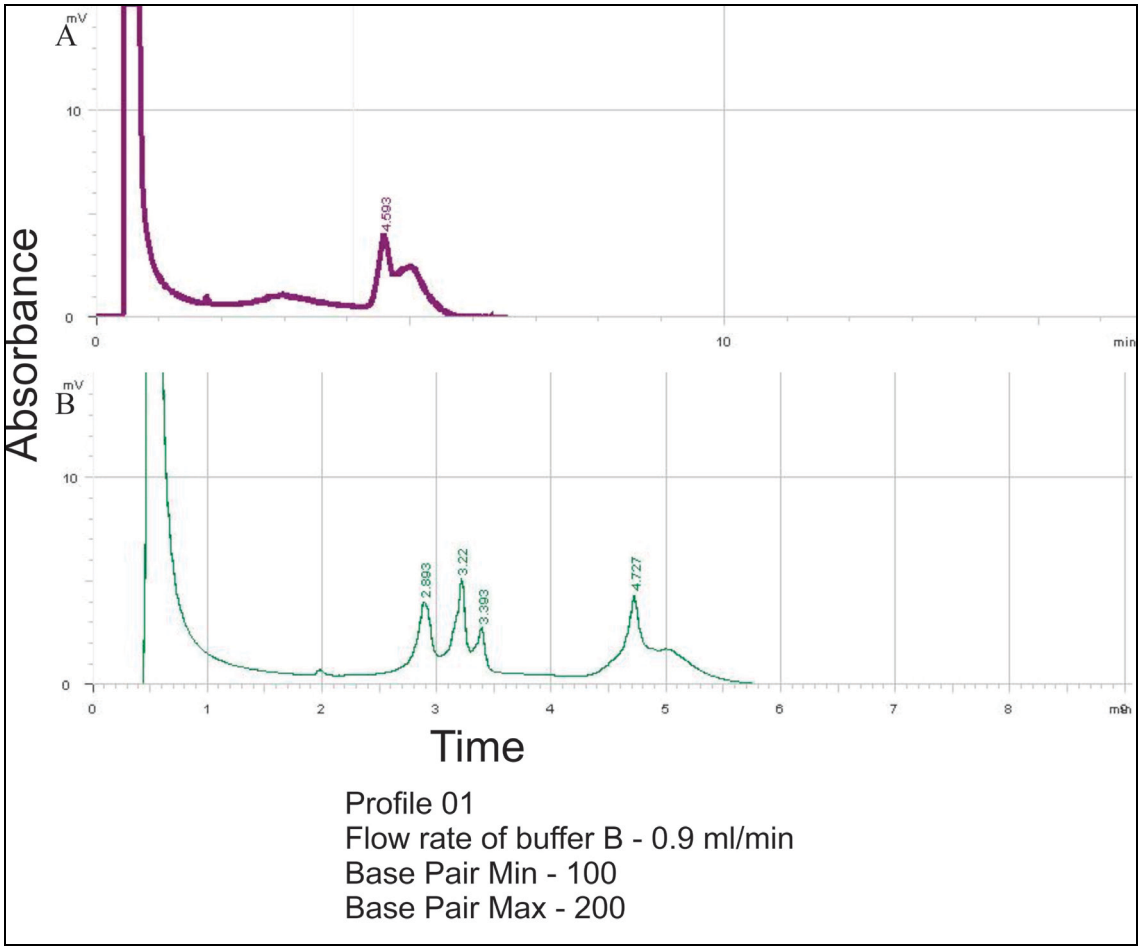


Figure 19:- Standardization of vWA loci. A- The DHPLC chromatogram of vWA locus using buffer profile 1 at 58 ^{0}C. B- The chromatogram at 50 ^{0}C using the same buffer profile. Flow rate, base pair minimum and bas pair maximum of profile 1 are given below the chromatogram.

As shown in **Figure 19**, partially denaturizing condition, chromatogram did not show well resolved peaks hinting that the STR alleles are not separated. Fractions were not collected at 58 ^{0}C. Whereas, fractions were well resolved at 50 ^{0}C, as shown in **Figure 19B.** Thus it is standardized that the alleles corresponding to vWA loci could be isolated at an oven temperature of 50 ^{0}C for the above specified buffer profile at a range of 100-200 bp. The fractions were collected for sequencing.

4.2.2.4. DNA sequencing data allelic fractions collected for vWA locus

The DHPLC fractions for sequencing were collected from the peaks corresponding to profile for 100-200 base pair range of vWA loci at 50 ^{0}C. Sequence data is shown in **Figure 20**.

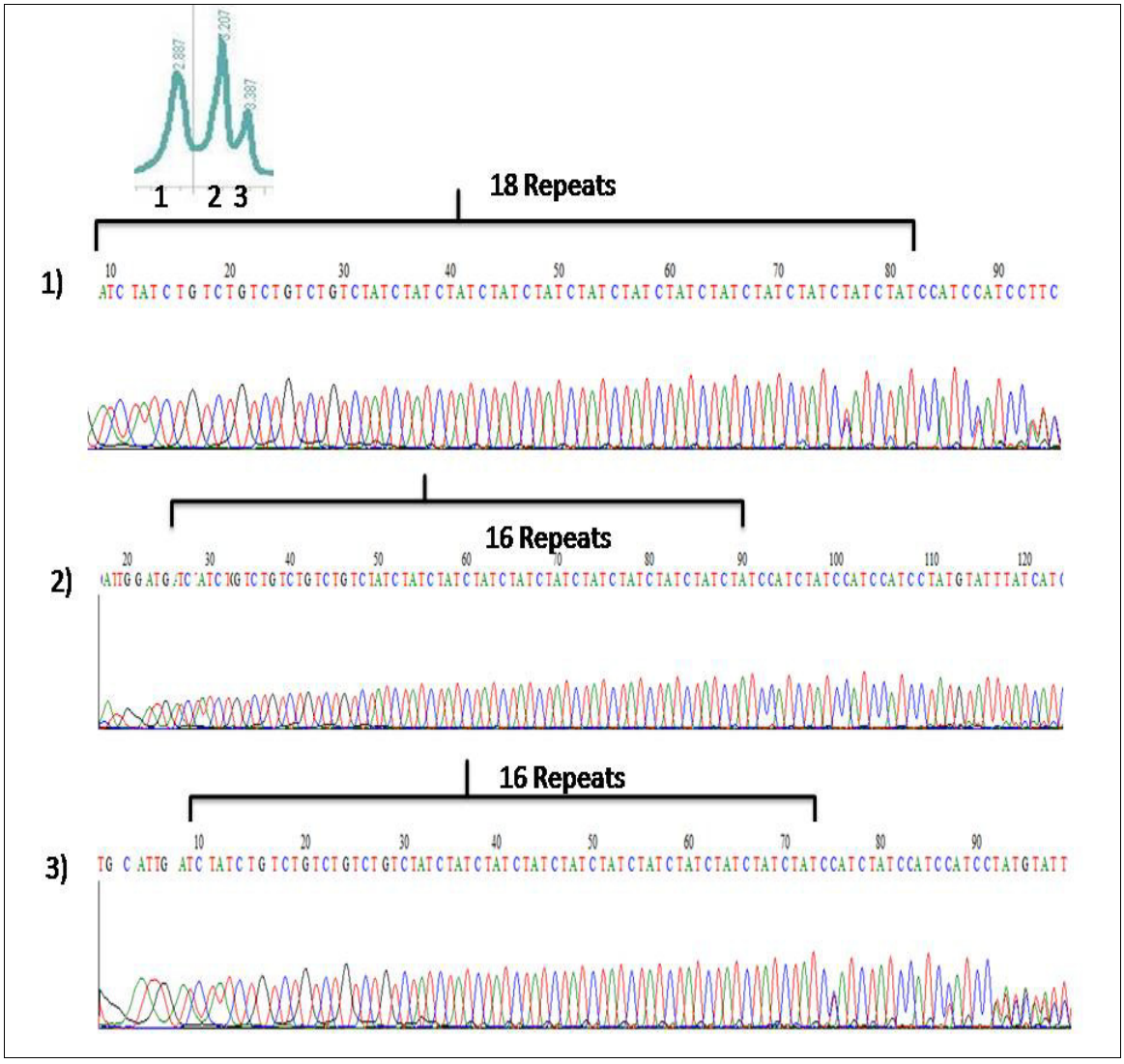


Figure 20:- DNA sequence data of allelic fractions collected for vWA locus. A. Peak corresponding to fraction collected numbered 1, 2 and 3. The sequence corresponding to each fractions obtained by DNA sequencing are numbered accordingly. The fraction 1, corresponds to peak 1 shows 18 repeats. The fractions 2 and 3 on sequencing showed 16 ATCT repeats.

In vWA loci also, peak 3 seemed to be similar to peak 2 with a sequence variation only at flanking region and not at the STR region as the number of repeat units being the same in both the fractions. Both the fractions have 16 repeats of sequence TCTA. Fraction 1 revealed 8 repeats of ATAG.

4.2.2.5. Standardization of allelic separation of D16S539 locus using DHPLC

The D16S539 locus is 264-304 bp in size. The PCR product containing amplified D16S539 locus was run in 2 different buffer profiles corresponding to 2 base pair ranges

entered. The flow rate was maintained at 0.9ml/min in all the runs. The 2 different profiles used are described as follows.

1. Buffer profile for 200-310 base pair range

   -Temperature: 58 ^{0}C

2. Buffer profile for 100-310 base pair range.

Well resolved peaks were not observed using profile 1 at 58 ^{0}C and hence fractions were not collected. At 50 ^{0}C four peaks were resolved against the 2 peaks expected (**Figure 21**). Even though the resolved fractions were collected; the standardization was continued for a wider base pair range 100-310 instead of 200-310 to avoid separation of alleles based on minor sequence changes

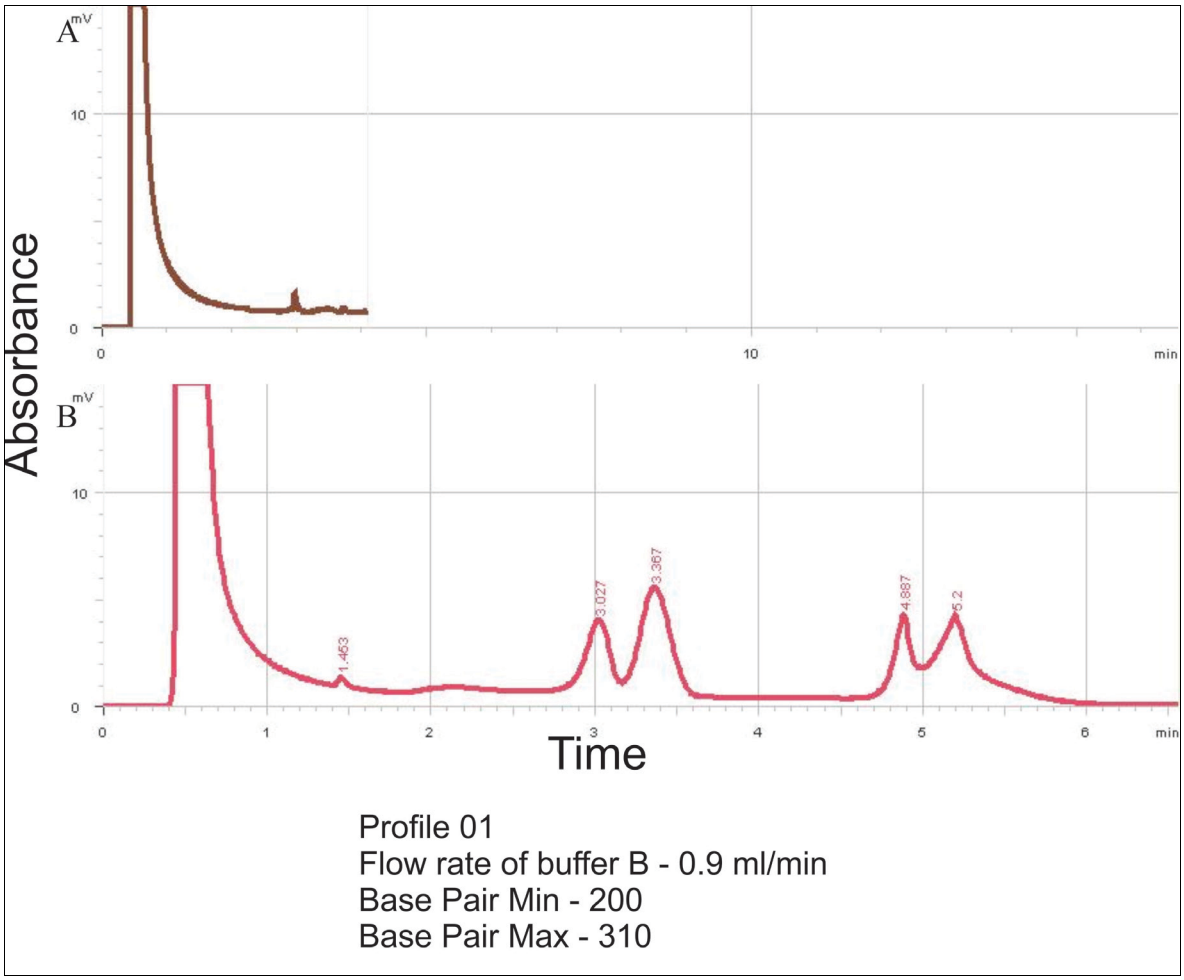


Figure 21:- Standardization of D16S539 locus (buffer profile 1). A- DHPLC chromatogram of D16S539 locus amplicon using the buffer profile 1 at 58 ^{0}C, B- chromatogram of D16S539 locus amplicon using the buffer profile 1 at 50 ^{0}C, shows well

resolved peaks. Flow rate, base pair minimum and bas pair maximum of profile 1 are given below the chromatogram.

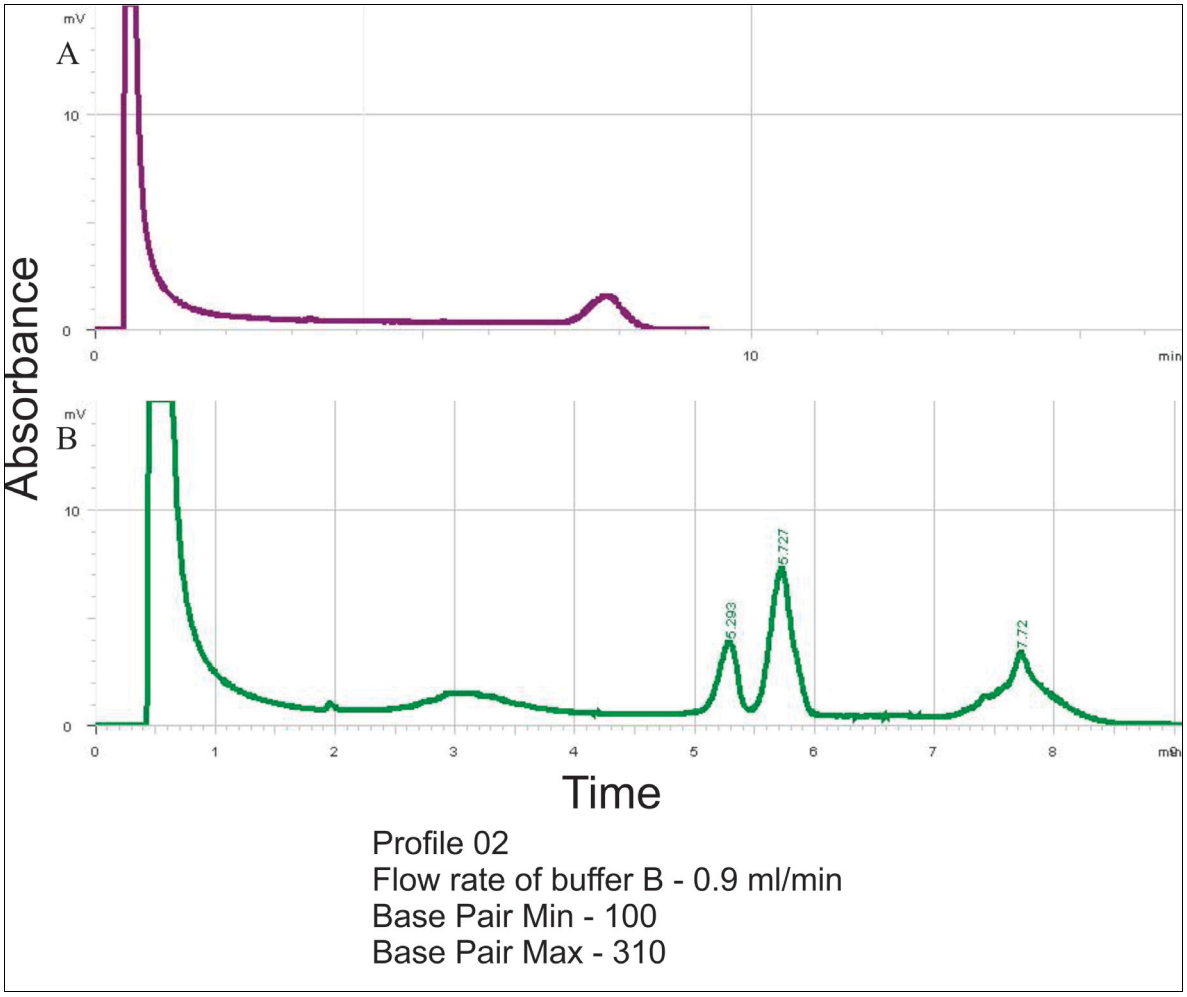


Figure 22:- Standardization of D16S539 locus A (buffer profile 2). A- DHPLC chromatogram of D16S539 locus amplicon using the buffer profile 2 at 58 ^{0}C; B- chromatogram of D16S539 locus amplicon using the buffer profile 2 at 50 ^{0} C, shows well resolved peaks. Flow rate, base pair minimum and bas pair maximum of profile 2 are given below the chromatogram.Well resolved peaks were observed at 50 ^{0}C using the profile 2 and 3, shown in **Figure 22** indicating proper STR allelic separation. Fractions corresponding to peaks were collected for sequencing.

4.2.2.6. DNA sequencing data allelic fractions collected for D16S539 locus

The DHPLC fractions for sequencing were collected from the peaks corresponding to profile 3 for 100-310 base pair range of DI6S539 loci at 50 ^{0}C. Sequence data is shown in **Figure 23.** Sequencing of the fractions collected for D7S539 locus revealed that the peaks

1& 2 having 9 and 10 repeats respectively of sequence CTAT. The wide base pair range selected for the loci would have helped in obtaining the discrete fractions.

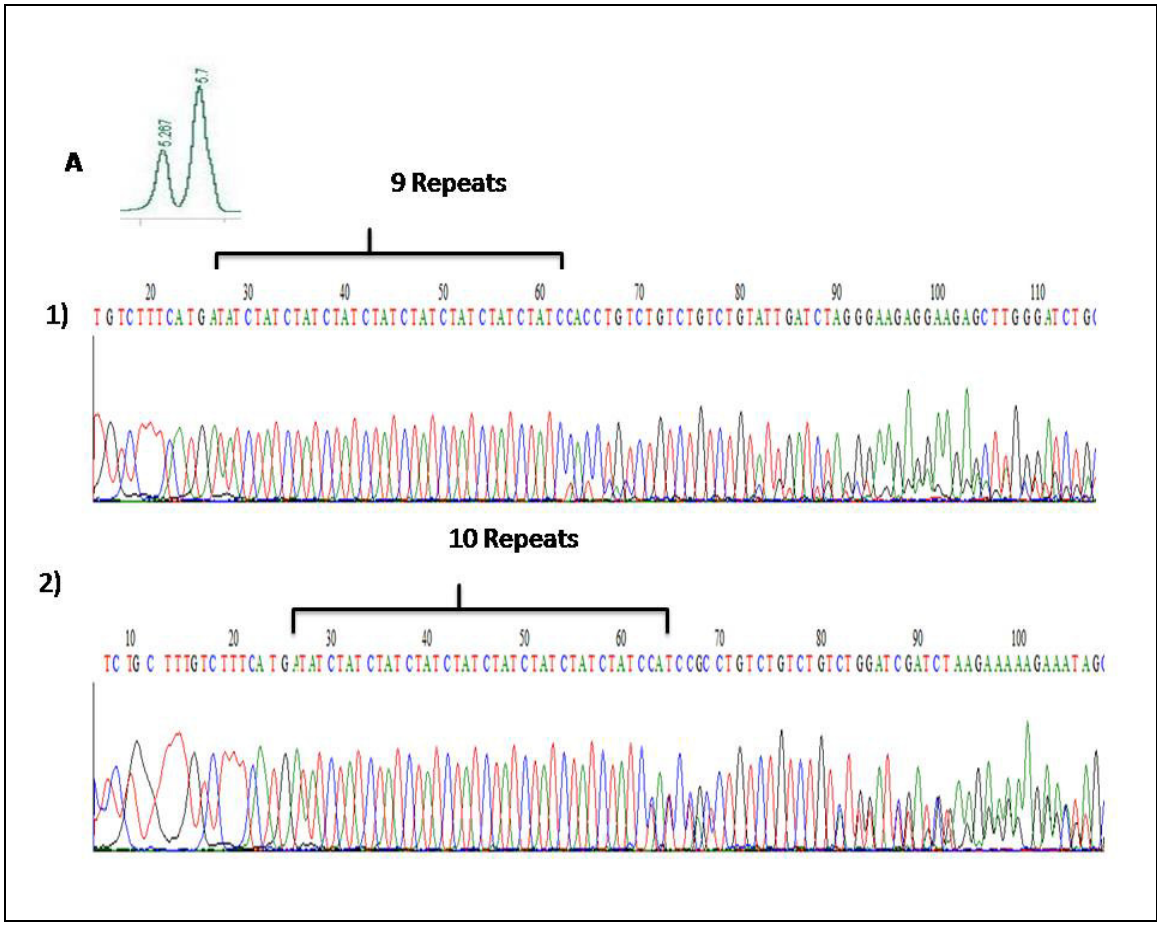


Figure 23:- DNA sequence data of allelic fractions collected for D16S539 locus. A- Peak corresponding to fraction collected numbered 1and 2. Sequence corresponding to each fraction obtained by DNA sequencing is numbered accordingly. The fraction 1 shows 9 repeats and fraction 2 shows 10 repeats of ATCT.

Thus it is deduced that the separation of DI6S539 alleles using DHPLC requires an oven temperature of 50 ^{0}C, using the buffer profile 2 with a wider base pair range, at a flow rate of 0.9 ml per minute.

4.2.2.7. Comparison of STR profile of HEp-2 from current study with ATCC HEp-2 profile for the locus studied

The number of repeats obtained from the study was compared with ATCC STR profile data for HEp-2 cells and summarized in **Table 5.** From the study, it can be deduced that

temperature coupled with buffer profile ensures effective separation of STR alleles from PCR amplicons.

TABLE 5: **Comparison of STR profile of HEp-2 from current study with ATCC**

STR region	Standardized DHPLC oven temperature (in ^{0}C)	No of STR allelic fractions from DHPLC	No of repeats observed after sequencing	ATCC STR profile for HEp-2 cell line
Vwa	50	3	16, 18	16, 18
D16S539	50	2	9, 10	9, 10
D7S820	50	3	8, 12	8,12

4.2.3. Validation of sterilization/disinfection of the polymer by ETO and ethanol by MTT assay

The ETO sterilization resulted in decrease in viability of both HEp-2 (36%) and Vero (85%) cells, where as ethanol disinfection did not affect the viability of both the cells as shown in **Figure 24**.

EtO is an alkylating agent. Even though it ensures complete sterilization compared with ethanol, which only disinfects, It has been reported that EtO has cytotoxic effects also. Based on concentration, EtO exerts various effects like disruption of cells, precipitation of cell protein, inactivation of enzymes and leakage of amino acids from the cells (111).

4.2.4. Cell Viability Assay using continuous cell lines

Biocompatibility of the matrix in pure form as well as in combination with PEG, PLA, and MMT was analyzed based on MTT assay with respect to continuous cell line (HeLa, HEp-2, Vero and McCoy) (**Figure 25)**. Cellular viability was superior in PHA blend with PEG with a p value ≤ 0.001 as tested by Tukey test. Blends with MMT showed a significant decrease in viability for all the cell types.

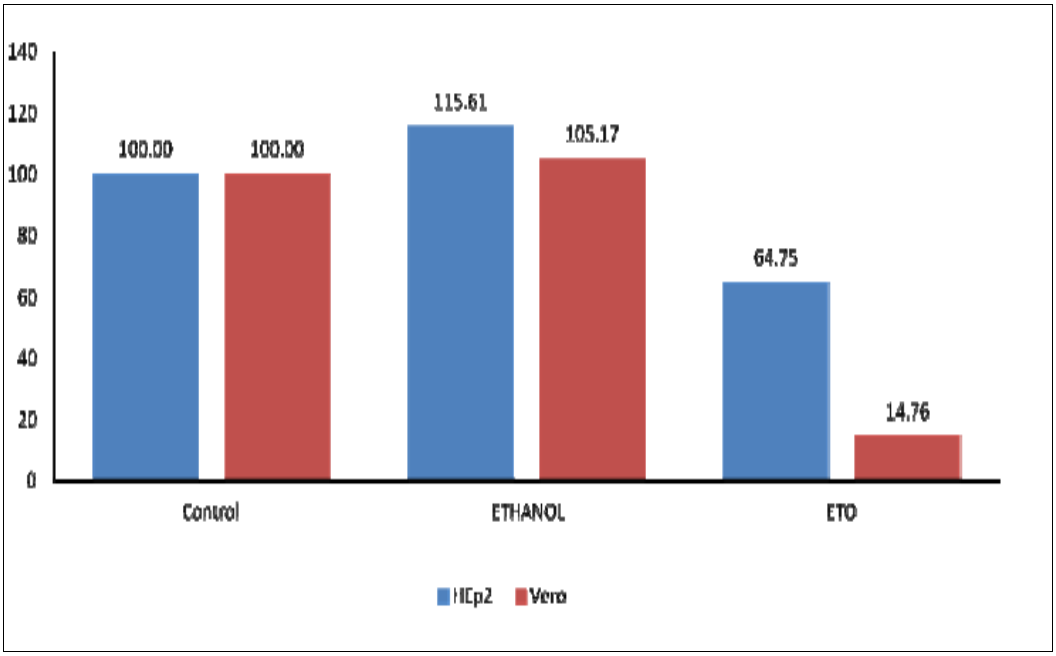


Figure 24:- **Evaluation of cytotoxic effect of sterilization.** The effect of EtO and ethanol sterilization of the PHA: PEG on cellular toxicity as measured by MTT assay. ETO sterilization resulted in decrease in viability of both HEp-2 (36%) and Vero (85%) cells, where as ethanol sterilization did not affect the viability of both the cells.

4.2.5. Proliferation assay

Proliferation rate was analyzed by colorimetric cell proliferation assay by ELISA (Bio tech power wave XS) as shown in **Figure 26**. There was 16, 31 and 23 percentage increase in proliferation of HeLa, McCoy and Vero respectively on the matrix PHA: PEG and was statistically significant ($p \leq 0.05$) when compared with the respective controls on 96 well plate surfaces. Statistical significance of the results was calculated using ANOVA followed by Tukey test.

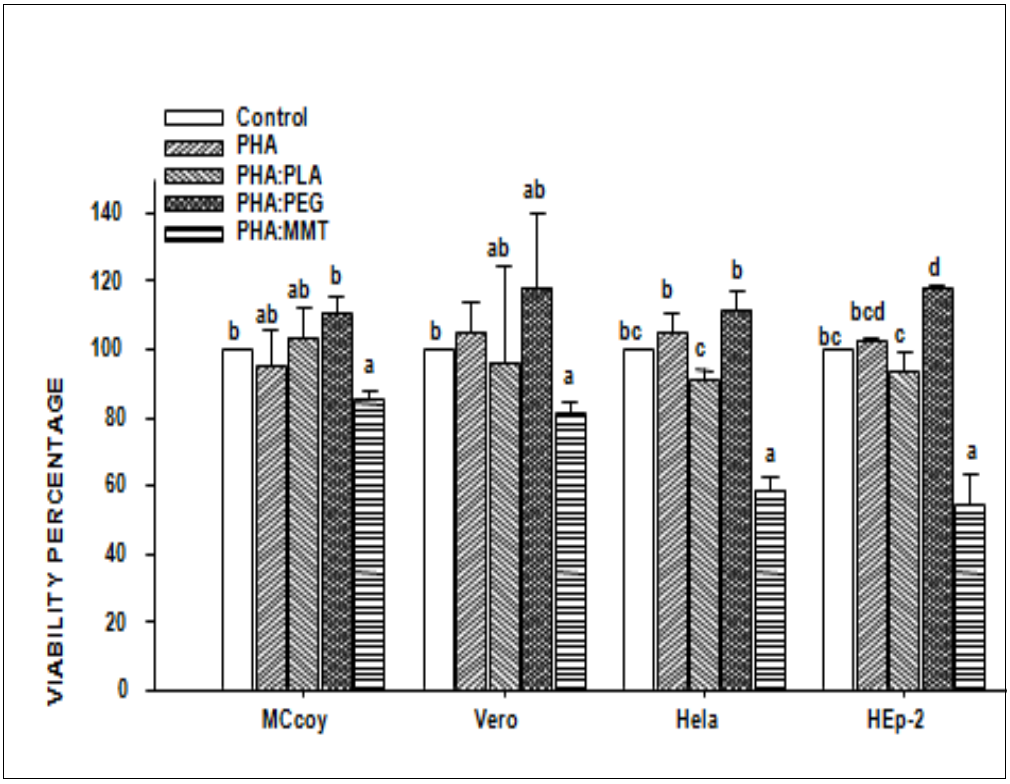


Figure 25:- Cellular cytotoxic assay for continuous culture by MTT at day 2. Each bar represents the arithmetic mean value of 3 replicates ± standard deviation. Statistical differences ($P<0.001$) tested by Tukey test among different biomaterial are indicated by different letters. Within each parameter tested, similar alphabets indicate no significant difference among biomaterial used.

4.2.6. Cellular morphology analysis

4.2.6.1. Phase contrast microscopy

Cellular morphology was observed by Phase contrast microscopy and the images are shown in **Figure 27**. Cells grown were compared to that of controls over the plate. Morphologic similarity was prominent in controls and matrix irrespective of the cell types.

4.2.6.2. Electron Microscopy

Cellular morphology of HeLa and McCoy cells grown over PHA: PEG was observed by SEM and the images are shown in **Figure 28**. From SEM images it was evident that the cells were infiltrated into the pores of PHA: PEG, pointing that the polymer has a potential to support 3d cultivation of cells.

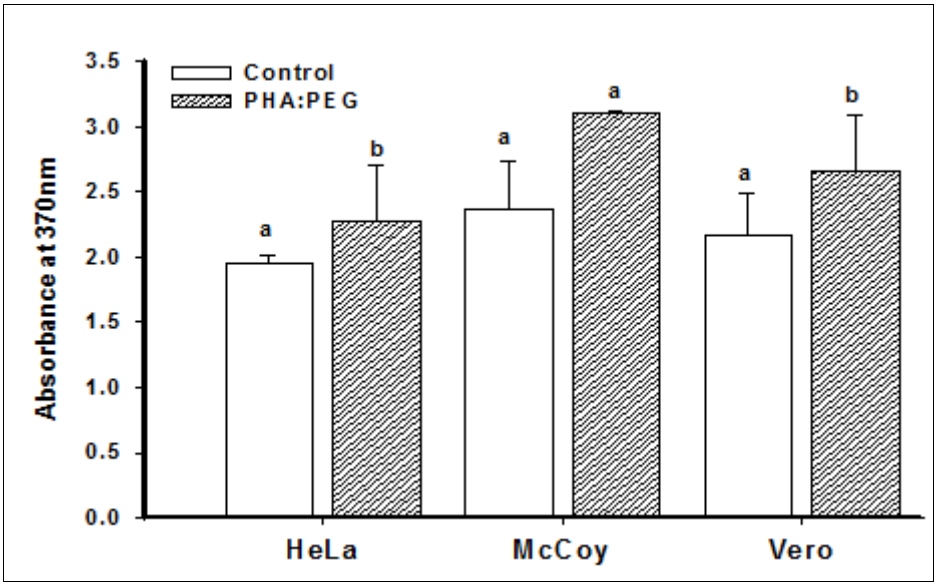


Figure 26:- Cell proliferation assay of HeLa, McCoy and Vero on PHB: PEG and control cell culture plate by BrdU ELISA. Statistical differences ($P<0.05$) tested by Tukey test among different biomaterial are indicated by different letters. Within each cell line tested, similar alphabets indicate no significant difference between the biomaterial used.

4.3. Evaluation of polymer for limbal cell cultivation

4.3.1. Corneal limbal explant culture and evaluation of outgrowth

Limbal biopsies were placed on the matrix as shown in Figure 30 A. Limbal cell outgrowth was observed within 24 hours by phase contrast microscopy. The cells were round with high N/C ratio. Cells were proliferating and after 5 days cells started differentiating towards the periphery of the culture (**Figure 29. B, C and D**) leaving the stem cell population around the tissue

4.3.2. Evaluation of Sections stained with H&E and DAPI

Vertical sections of the scaffold with the culture showed 3 layers of cells when stained with H & E (**Figure 29 E**). This indicates that cells were differentiated and stratified. Fluorescent staining of the nucleus by DAPI supported this observation (**Figure 29 F**). Stratified layers of the cells were embedded within the matrix. This might be due to the penetration of cells through the pores of the matrix.

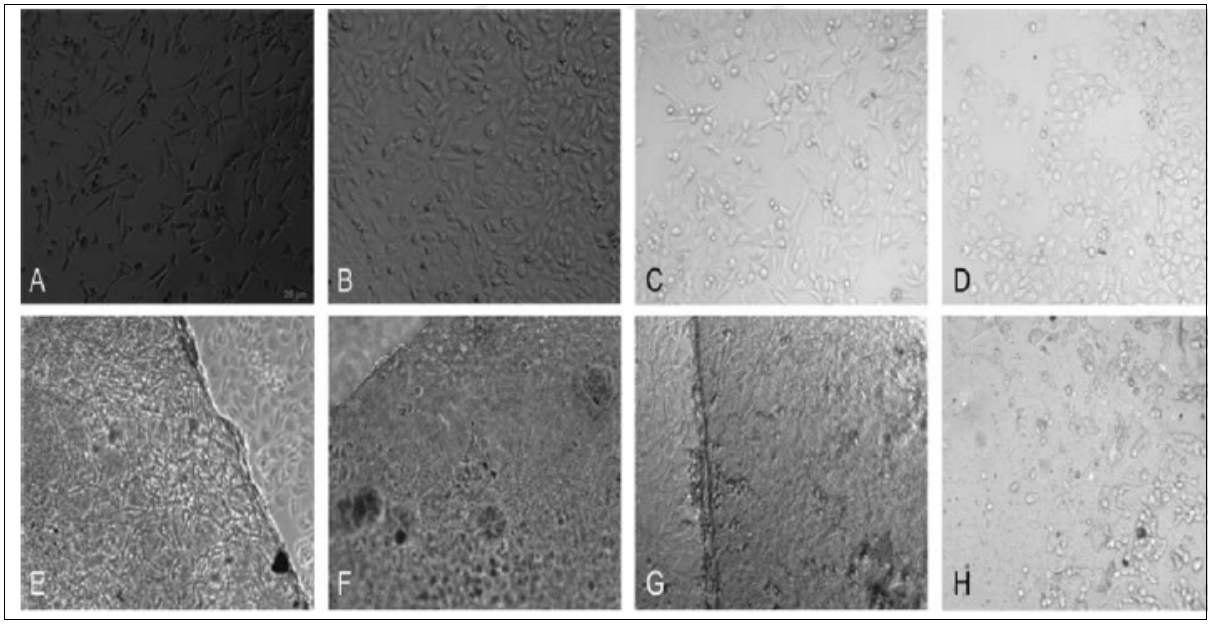


Figure 27:- **Morphology of cell cultures on the matrix.** A to D- Control culture; E to H- culture on matrix. A & E- McCoy; B & F- Vero; C& G- HEp-2; D & H- HeLa. All the images are taken at 10 X magnification in Carl Ziess Axio observer Microscope.

4.3.3. Rate of corneal limbal epithelial cell proliferation on PHA: PEG scaffold compared to control tissue culture surface

Prolifertion of corneal limbal epithelial cells on the PHA: PEG scaffold was analysed from day 1 to day 3 in comparison with that on tissue culture plate. As shown in the Figure 2, proliferation rate of corneal limbal cells, at day1, day2 and day 3 showed a higher rate ofproliferation on the polymer PHA: PEG compared to the culture plate. The rate of proliferation showed a 39% increase on PHA: PEG at 3rd day of cultivation (**Figure 30)**.

4.3.4. Expression of corneal limbal epithelial marker by qualitative RT- PCR

By qualitative RT- PCR, it was shown that the putative stem cell markers ABCG-2 and NP-63 were expressed at early days of culture (0-2 days). At later stages of the culture expression of the same was not clearly seen by qualitative real time whereas, corneal epithelial differentiating markers *K3*, *K12*, Cx43 were clearly expressed (as shown in **Figure 31)**

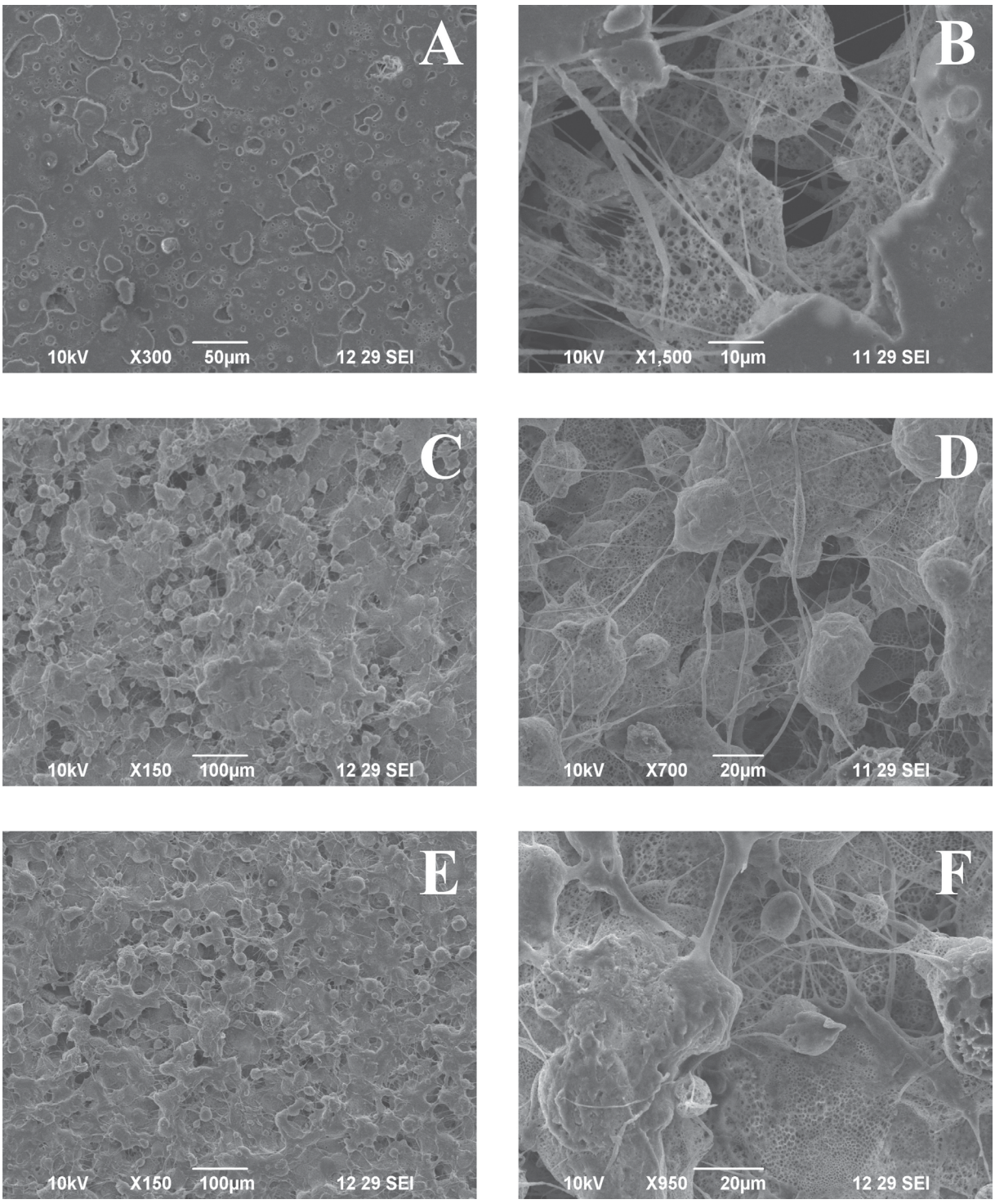


Figure 28:- **Morphology of cell cultures on the matrix by SEM.** A& B- Control Matrix PHA: PEG; C & D- HEp- 2 culture on matrix; E & F- McCoy culture on matrix. A, C & E are low resolution and B, D and F are high resolution images.

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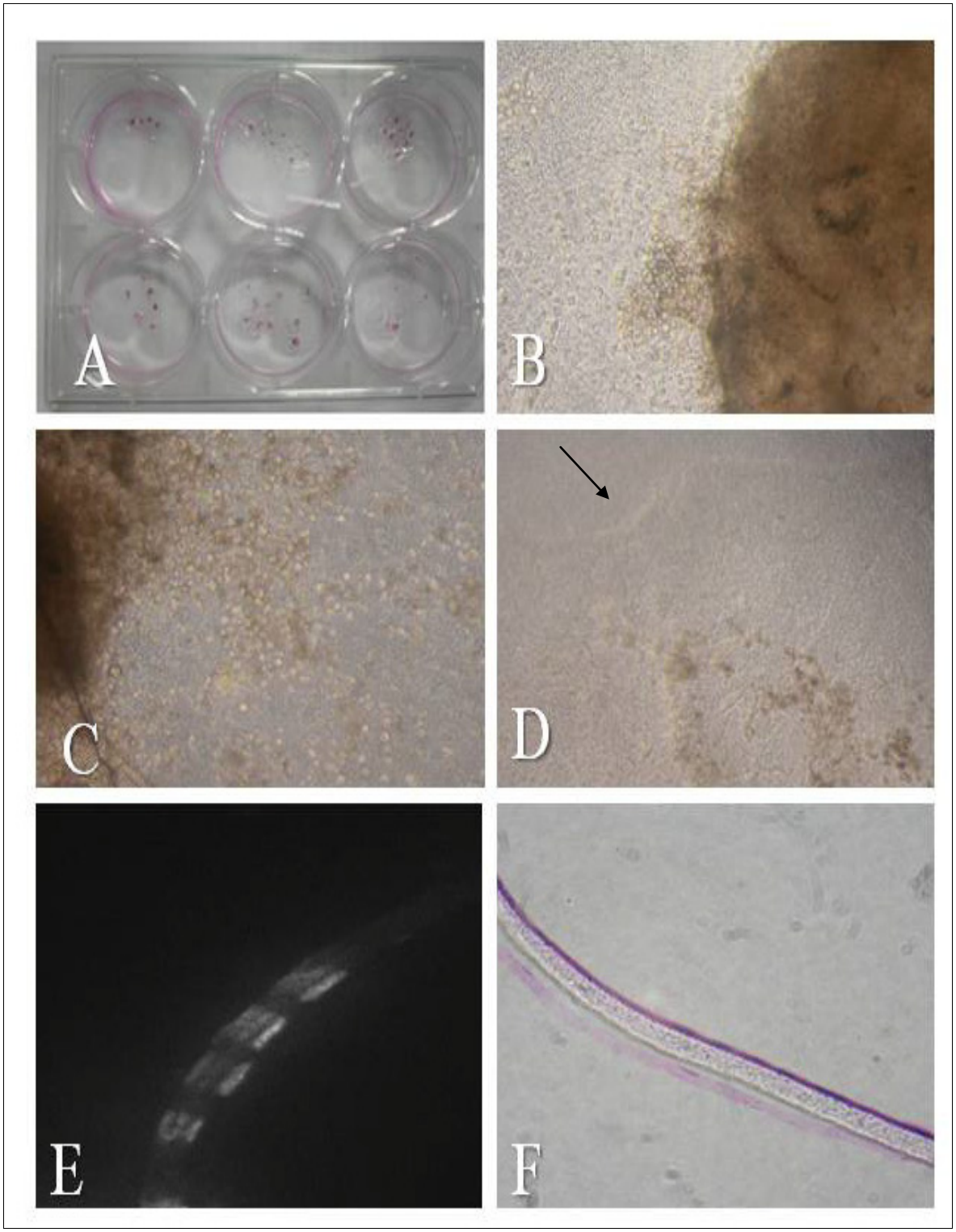

Figure 29:- Corneal limbal stem cell culture. A- Limbal tissue explanted over the scaffold; B- Outgrowth of cells within 24 hours (10 X phase contrast microscopy); C- Limbal stem cell proliferation at day 2 (10 X phase contrast microscopy); D- Differentiated corneal epithelial cells at day 5 (10 X phase contrast microscopy); E- H & E staining at

Day 12 shows 3 layers of differentiated cells (40X bright field); F- DAPI stained nuclei of stratified epithelium (40 X fluorescent microscopy).

4.4. Comparative evaluation of the morphology of cells grown in various matrices

4.4.1. Evaluation of Cellular Morphology

Cellular morphology in each system was analyzed by Carl Ziess axio observer microscope and phase contrast images are shown in the **Figure 32**. All the gel based systems formed spherules of the cell. Spherule diameter was more for mebiol gel than the other gel systems. Whereas, Hydromatrix peptide hydrogel formed small spherules. In control 2d culture and PHA: PEG cells appeared as a layer and did not form spherules (**Figure 32 A-E).**

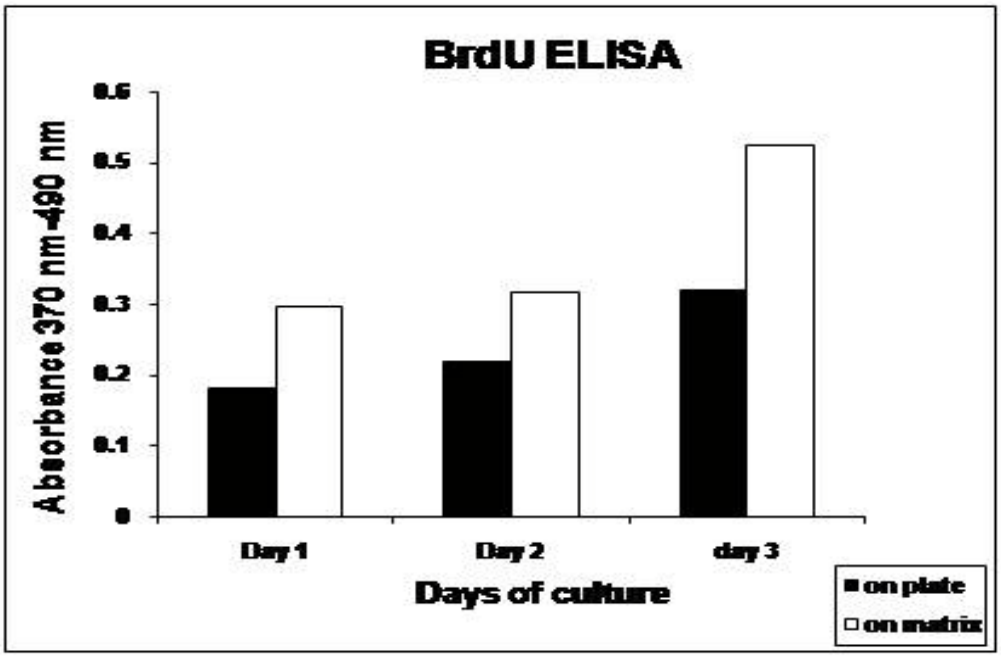

Figure 30:- BrdU Cell proliferation ELISA. A higher rate of proliferation of corneal limbal epithelial cells on the PHA: PEG scaffold was analysed from day 1 to day 3 in comparison with that of tissue culture plate.

4.4.2. Evaluation of cellular proliferation

Cellular proliferation in each system was analyzed by BrdU ELISA at 48 hours of cultivation. The Figure shows the rate of multiplication in each system. The cell proliferation was significantly higher in hydromatrix system followed by PHA: PEG,

Matrigel, Mebiolgel and control (**Figure 32 F**). Statistical significance was done by student's t test.

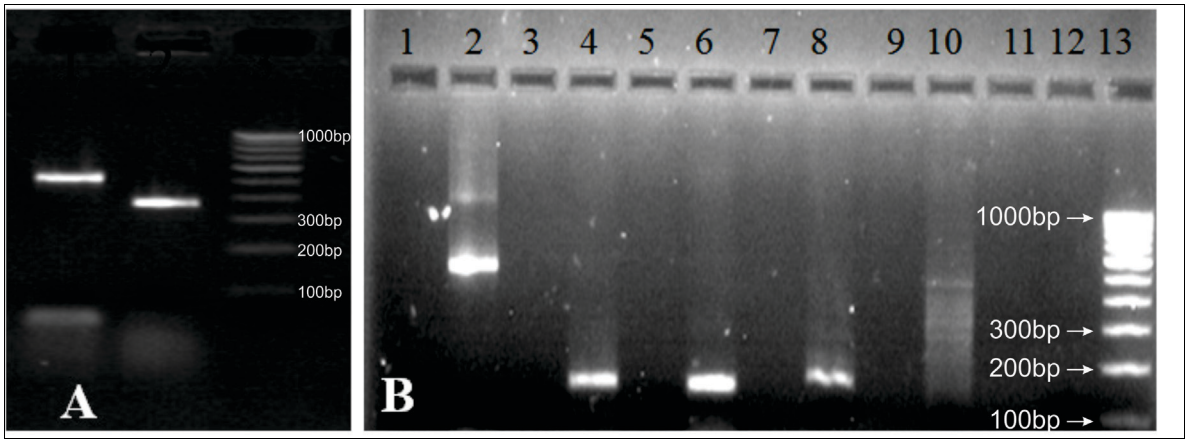

Figure 31:- Expression of putative Corneal limbal epithelial stem cells and differentiating epithelial cells. A- Expression of Np-63 (lane 1) ABCG -2 (lane 2) at day 2; B- Expression of *GAPDH* (lane 1) *K3* (lane 2), *K12* (lane 3), CX43 (lane 4), (Np -63 lane 5) at day 12**.** Lane 13 is 100 bp ladder. Lanes 1,3,5,7,9,11 and 12 were left blank.

4.5. Evaluation of the scope of different polymer systems to support viral multiplication

4.5.1. Evaluation of titer of stock virus by plaque assay

The number of plaques visualized by the end of third day was counted and multiplicity of infection was calculated as follows.

Plaque forming unit /l00 µl = Number of plaques formed dilution factor

Multiplicity of infection per cell is calculated as follows

Multiplicity of infection (MOI) = Plaque forming unit /ml/number of cells plated

The stock virus was then diluted to get an MOI of 0.5 for further analysis.

4.5.2. Evaluation of morphology of HCE cells before and after infection

Control HCE showed CPE, by infecting with 0.5 MOI HSV 1 stock virus, within 12 hours. Cells on the periphery of plate started peeling off at the end of 24 hours and hence could not be incubated further. Cells in all the polymer gel systems formed spherules before infection and on infection, spherules showed distorted appearance only after 24 hours as seen in **Figure 33.**PHA: PEG showed spreading of cells before infection as in control.On infection it showed a CPE within 12 hours and the morphology of the cells could be studied till 48 hours.

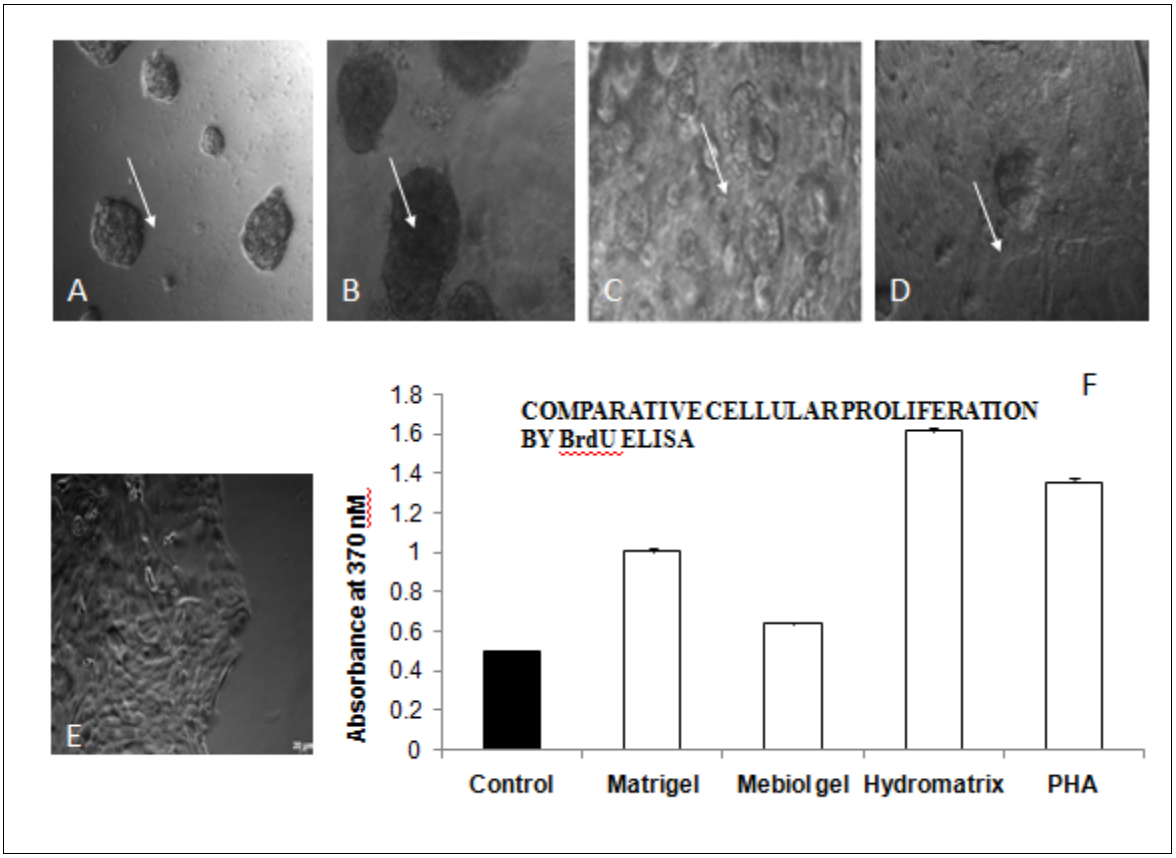

Figure 32:- **Evaluation of Cellular Morphology and Rate of proliferation of HCE in different matrix systems.** A- HCE on matrigel (from *invitro*gen); B- HCE on mebiol gel (from NCRM); C- HEp-2 on Hydrogel (Sigma); D- HEp-2 on PHA: PEG (indigenously produced) E: HCE on plastic surface; F- Chart showing comparative evaluation of cellular proliferation of BrdU ELISA.

4.5.3: Evaluation of the rate of multiplication of HSV-1 in different polymer systems

The HSV -1 count in supernatant of each system after 48 hours was done by HSV specific real time PCR and was compared. The count was normalized with the initial count of HSV-1 which was used for infection. The results indicated **(Figure 34. I)** that PHA: PEG has a maximum multiplication of virus with almost double as that of control, and mebiolgel showed the least. HSV multiplication in matrigel was slightly higher than that of control and in hydrogel it was slightly less than that of control. Hence it was evident that the system in which cells grow influences the rate of multiplication of HSV.

4.5.4. Relative expression of TLR in response to HSV infection by real time PCR

The expression of TLRs 1, 2, 6 and 9 were evaluated in each system and normalized with the infected control 2d samples which was obtained by normalizing with the expression of

uninfected control samples.As shown in **Figure 34. II**, there was a better TLR response in cells grown on Hydro matrix peptide gel in comparison with other systems even though all the polymer system exhibit better TLR response compared to 2d culture systems. TLR1, 2, 6, and 9 were less expressed in PHA and mebiolgel whereas Hydromatrix and matrigel system showed upregulated expression of TLRs. Hydromatrix system showed 1900 fold increase in expression of TLR2, 1400 fold increase in TLR6 and 424 fold increase in TLR9. Whereas matrigel system showed an upregulation of TLR2 and TLR6. Up regulation of TLR6 expression in HCE in response to HSV-1 has not been reported in literature to the best of our knowledge

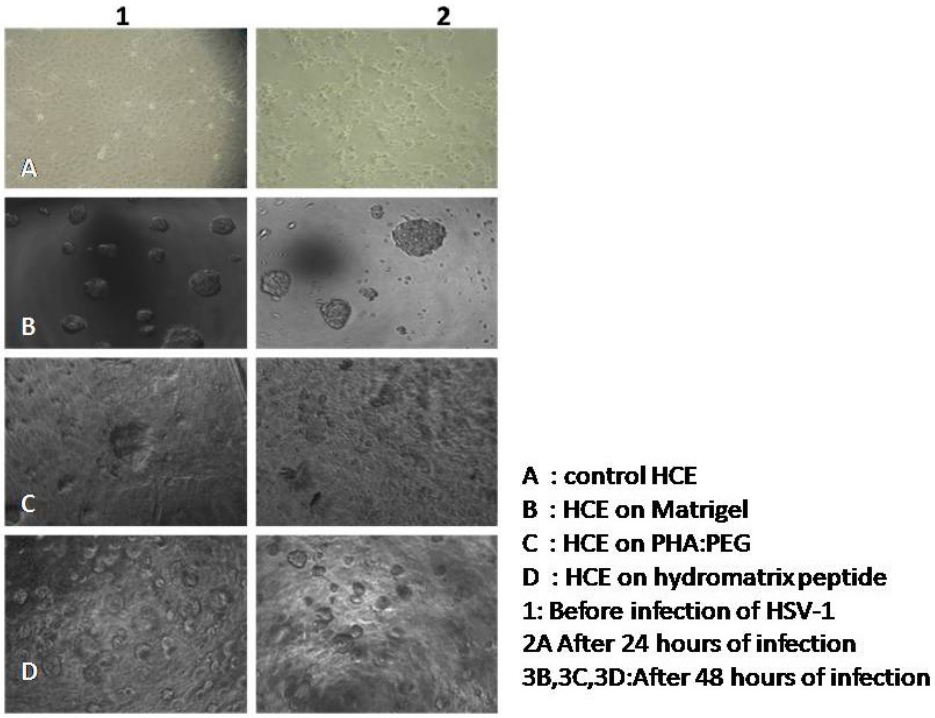

Figure 33:- HSV-1 infectivity in various matrices by Morphology- Morphology of HCE before and after infection. A1-Morphology of control HCE before infection and A2 is corresponding change (CPE) after infection; B1-Morphology of HCE on Matrigel before infection and B2 represents the same after infection; C1-Morphology of HCE on Hydromatrix before infection and C2 shows CPE of the same after infection; D1-Morphology of HCE before infection on hydromatrix peptide hydrogel system and D2 shows the same after infection.

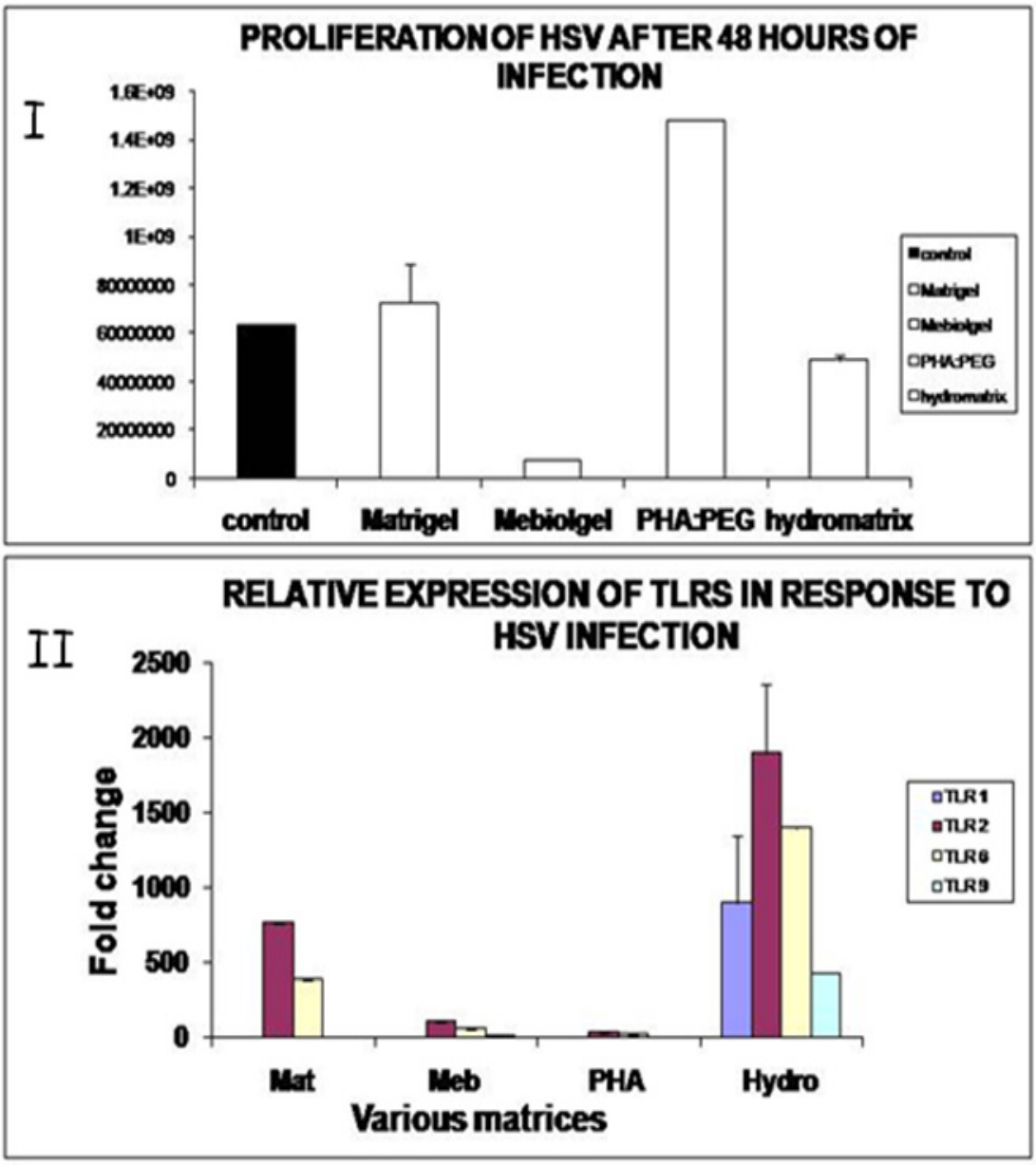

Figure 34 - HSV-1 infectivity study by proliferation and immune response: I: Chart shows the comparative rate of multiplication of HSV in the four systems studied; **II**- Chart represents the comparative fold change of expression of the four TLRs in response to viral infection in each system.

TABLE 6: Comparative evaluation of HSV-1 infectivity in different polymers compared to control

Polymer	Morphology of HCE	Cellular Proliferation rate by absorbance	Viral multiplication in terms of difference in viral count	TLR response Fold change
PHA	Comparable with plate culture	1.3	2.33	TLR1- 1.9 TLR2- 36.23 TLR6- 19.26 TLR9- 6.46
Mebiogel	Cells grow as spherules	0.67	0.16	TLR1- 7.01 TLR2-108.83 TLR6- 57.92 TLR9- 11.62
Matrigel	Cells grow as spherules	1.0	1.3	TLR1- 2.9 TLR2-768.36 TLR6-385.64 TLR9- 0.569
Hydromatrix	Cells grow as spherules	1.6	0.83	TLR1- 99.50 TLR2-1900 TLR6- 1400 TLR9- 424

CHAPTER 5

DISCUSSION

Polymers used for cell cultures should be biocompatible besides providing a suitable substrate for cellular proliferation and differentiation. Various natural materials have been studied for this purpose and it includes extra cellular matrix (ECM) proteins such as collagen types I, III and IV,fibronectin, laminin, gelatin, and vitronectin, thermo reversible polymers; Robertson and polylysine (109, 112). The above listed materials, are however associated with disadvantages such as inhibition of cell proliferation, limited availability and poor processing properties. As PHA is completely biodegradable, biocompatible and biorenewable, it has been considered as a good candidate for this purpose. Here, for the first time PHA recovered from *Pseudomonas* sps *MNNG-S*, (Polyhydroxy propionate co-poly hydroxy ocatadecanoate co-polymer), was used for the cultivation of cells in the current study.

In order to improve the processing properties and to construct a transparent thin film, the pure polymer was blended with other biodegradable polymers like PEG, PLA and MMT. Solvent casting method is the simplest and finest technique that helps to fabricate scaffolds with desired pore size. As the pore diameter of the scaffold was around 40 μm by electron microscopy, it is suitable for cellular infiltration and permits cell-cell interaction (113). The polymer purity was ascertained as it is of microbial origin and it did not contain any other contaminating agents or endotoxin as tested. Blending of PHA was standardized at a proportion of 60:40 with PLA, PEG and MMT to select the best biomaterial for *in vitro* cellular growth. Among all blends, PHA: PEG at 60:40 formed a thin transparent film and it was more biocompatible with continuous cultures.

Protein adsorption onto polymer surfaces has been known to be strongly affecting the cellular interactions with foreign surface (4). This helps to induce the macrophage activation followed by immunologic response to foreign materials (109) and thus, control the adhesive behaviour of the cell. In this study, PHA blended with PEG exhibited maximal protein adsorption. BSA (protein) adsorption on a surface of an adsorbent at a neutral pH occurs due to electrostatic interactions. Since BSA is negatively charged at neutral pH and the blends fabricated using different conditions also exhibited a negative surface charge at neutral pH, other interactive factors such as van der Waals, and steric interactions could have also contributed to the protein-adsorbent interaction (4). The result

of cellular attachment and spreading is explained by the physiochemical properties of the material. The first non-specific adsorption of cells on material is mainly mediated by hydrophilicity, which contributes to the physio-chemical interaction (112). From all the experiments performed, PHA: PEG matrix serves as a better one owing to improved hydrophilic property.

The solvent casted PHA: PEG scaffold is not cytotoxic as indicated by MTT assay with HeLa, HEp-2, Vero and McCoy. Phase contrast analysis reveals the cellular adhesion and spreading on PHA: PEG matrices**.** This is in accordance with reports of studies favoring the cell attachment on film surface with parallel groves (112). An increase in cell viability indicates cell growth, while a decrease in viability is interpreted as a result of either toxic effects of compounds or suboptimal culture conditions. This assay inferred that the prepared blends (PHA: PLA, PHA and PHA: PEG) are non-toxic and are compatible, hence have led to proliferation of cells indicated in terms of increased cell viability. Increased rate of proliferation of cells on PHA: PEG compared to the cells on conventional plastic surface with the BrdU ELISA shows that the material produce better cell adhesion and communication compared to conventional cultures.

The experiments with continuous culture strongly supported the fact that PHA: PEG was superior in its properties and biocompatibility. Encouraged by this, we further studied the possibility of the PHA: PEG blend in *ex vivo* corneal limbal expansion. The epithelial layer of the cornea is on continuous cell-turnover where, the limbus has been shown to be the eventual source of epithelial regeneration (114). Considerable hurt to these cells causes a state called LSCD, which is characterized by varying extents of conjunctival overgrowth onto cornea, poor epithelial integrity and vascularization. This happens in case of microbial infections, chemical and thermal injuries and diseases such as Stevens - Johnson syndrome (115). Tseng and colleagues demonstrated that limbal stem cells can be used to reconstitute the entire corneal epithelium (116). This technique is known as limbal stem cell transplantation and has restored the eyesight of many patients. The technique involve, *ex vivo* expansion of autologous limbal epithelial stem cells using human amniotic membrane (HAM) as a carrier. HAM is used for corneal surface reconstruction because of its advantages of containing growth factor, anti inflammatory and anti angiogenic factors which prevents and decreases fibrosis in the healing tissue (21). Apart from the threat of infection, the use of feeder layer and complex medium containing growth factors adds on to the safety issue. Cultivation of limbal cells without any animal material has been a

challenge in LSCT (117). The cultivation of LESCs using animal-material free medium on HAM and on lens capsule (22) have been recently described for transplantation purposes.

Normally, outgrowth of limbal cells from limbal biopsies takes 12-24 hours with growth factors on amniotic membrane (116). It was striking to observe the same using PHA: PEG without any growth factors. The cells as it migrates started differentiating towards the periphery within 5 days of culture. Stratified morphology of corneal epithelium was visible in the H & E and DAPI staining of the vertical sections of the matrix. The differentiation of stem cells strongly depends on the availability of oxygen in time. Scientists have been manipulating the oxygen concentrations for cell culture and showed that lower oxygen tensions greatly influenced both embryonic and adult stem cell biology (118), (119), (120). This supported the faster differentiation of Corneal limbal epithelial stem cells. Markers for stemness and differentiation are mostly putative 'markers' which can either be present or absent. Basal cells of the limbus lack differentiation markers such as the 64 kDa cytokeratin 3 (C*K3*) that is present in the corneal epithelium and the suprabasal layers of the limbal epithelium (121). The corneal specific 55 kD protein, cytokeratin 12 (C*K12*) is also expressed in a similar pattern (122) Connexin 43 (123) and involucrin (93) both markers of cells destined for differentiation, are also absent. Sun and coworkers discovered in the early to mid-1980s that corneal epithelial cells synthesized two major tissue-restricted keratins called *K3* and *K12* (124). Using a monoclonal antibody AE5 to examine the expression of *K3* in cultured rabbit corneal epithelial cells, (121) noted that *K3* was associated with the upper, more differentiated, cell layers, indicating that *K3* was a marker for an advanced stage of corneal epithelial differentiation. When the expression of the *K3* keratin was examined *in vivo*, it was observed that this keratin was also expressed in the upper cell layers in corneal epithelium in the limbal zone; this was consistent with the concept that *K3* was a marker for an advanced stage of differentiation.

The transcription factor p63 is required for formation of epidermis and has been proposed as a putative positive LESC marker (125) . *In vitro,* p63 was found to be expressed in limbal epithelial cell derived holoclones with little or no expression in meroclones and paraclones. *In vivo*, p63 was located in the limbal basal epithelium. However, apart from these observations, a number of reports have suggested that p63 is not sufficiently specific to act as an LESC marker as it has also been localised to basal cells of the peripheral and central cornea in humans (93), (126). However, limbal epithelial cells expressing high levels of p63 with a high nuclear to cytoplasmic ratio appear to be more stem like (127) .

According to another study the isoform of NP 63, ΔNp63α may be a more specific label of LESC (128).

In the current study, ABCG- 2 and ΔNp63α positivity shows the stemness of cultivated limbal cells at early days (0-2) of culture. However, after 5th day of culture fully differentiated cells were seen towards the periphery. Lack of expression of stem cell markers during later stages of culture might be due to the less hypoxic condition of the tissue and cells on the polymer. The polymer, as it was very thin, was observed to be floating in the medium and ensured a good air– medium interface. Stem cell markers express well when the oxygen concentration is less and differentiating marker expression is enhanced by increased oxygen concentration (129). The decrease in expression of putative stem cell markers stands as a limitation of the study. Pore size (40 μm), obtained by solvent casting, can be brought down to nano dimensions by electrospinning method which might give a better corneal limbal cell proliferation and longer stemness by providing a more hypoxic environment.

Inspired by the success of continuous culture of cells on the polymer, the study progressed in the level of application of the same in developing a 3d system to study the infection and replication of viruses in general. As it was not practically possible to explore many viruses, with the constraints of time and money, the study used only one virus with a particular cell line to obtain a primary status of such a response. Among the infective viruses, HSV-1 was selected mainly based on the availability. Furthermore, there is no vital *in vitro* system to study the condition called viral keratititis for which HSV -1 is the major cause. The possibility of creating such an infective model for HSV was explored with Human corneal epithelial cells on PHA scaffold and compared with other commercially available polymers like matrigel, mebiolgel and hydromatrix peptide nanofiber scaffold. This study is novel as there is apparently no literature on a comparative study although individual commercially available polymers have been reported in creating a model for HCV.

Cellular proliferation and viral multiplication in each system was comparable except for hydromatrix based system. In hydromatrix based system, the HCE cells formed many smaller spherules which showed a significantly good cellular proliferation. However, the replication of HSV was not comparable with the cellular proliferation in this system. The results of TLR expression suggest that the decreased viral proliferation in hydromatrix peptide system would be due to the up regulated expression of TLRs. Among the HCE

specific TLRs, TLR2 and TLR9 were studied along with TLR1 and TLR6 in all the 4 systems. In this study TLR2 was highly expressed followed by TLR6 in HCE matrigel and hydromatrix system. Upregulation of TLR9 was significant only in hydromatrix system along with TLR1 even though TLR9 is reported to be upregulated in HCE in response to HSV infection in 2d culture system (130). The finding of up regulated TLR6 expression in hydromatrix peptide system is novel in HCE, in response to HSV-1. The matrigel system gives a similar but lower level of immune response to the infection.

The PHA: PEG system seems to be failed in offering a good immune response of the cells, in terms of the TLRs studied, to defend the infection. Along with this, the cellular stratification obtained in PHA: PEG would also have supported the enhancement of viral multiplication. Even though reports state that the mebiolgel forms a 3d cell culture system for HCV, this study could not make a good 3d system out of mebiolgel with HCE as both the cellular and viral proliferation was comparatively less in this matrix.

Another important achievement of the study is that it could standardize an STR fingerprinting method based on DHPLC for authentication of human cell lines. As the continuous cell culture, HEp-2 used in the study was a preserved stock in the laboratory, the authentication of the cell line before experiments became mandatory. Many methods exist for identification of human cell lines uses polymorphic repeat markers VNTRs and STRs for profiling. As decided in the ATCC-SDO workgroup in 2009, STR profiling is the method recommended by ATCC for cell line authentication (110). The existing methods for STR fingerprinting either use silver staining method which is less sensitive and cumbersome or gene scan application of DNA sequencing machine. Both of these use specific reagents supplied in a commercial kit form.

This study separated STR alleles from PCR amplicons using DHPLC. Sequencing of allelic fractions revealed the number of repeats in each allele. DHPLC based separation of STR alleles is not new in forensic science. Some of the STR regions that have been successfully separated using DHPLC include TH01 and F13A01 (87). In this study, three core STR loci from the FBI CODIS were isolated using DHPLC, namely vWA, DI6S539, D7S820 which have not been separated using DHPLC earlier. Furthermore the use of DHPLC based fingerprinting has not yet found a foot-hold in cell line authentication.

Most allelic separations are carried out under non-denaturing conditions (50 ^{0}C). However, the effect of partially denaturing conditions was also studied (58 ^{0}C and 60 ^{0}C). Non-

denaturing conditions yielded well-resolved peaks corresponding to STR alleles, while partially denaturing conditions either yielded a single peak of very low intensity or no peak at all. This may be attributed to the fact that applying high temperatures might lead to the loss of the product as there is no renaturation following the partial denaturation.

The study by Transgenomic for isolation of STR alleles from DHPLC employed a flow rate of 0.3ml/min (86). A higher flow rate of 0.9ml/min was employed in the current study. Higher flow rate resulted in faster elution of products, with no effect whatsoever on the peak intensity. From the study, it can be deduced that temperature coupled with buffer profile ensures effective separation of STR alleles from PCR amplicons.

By using DHPLC, PCR fragments can easily be purified from salts, unused dNTPs and unincorporated primers before the automated sequencing analysis. More so, sequencing becomes a lot easier as the STR regions have already been separated from the rest of the amplified region. Counting of tetranucleotide repeats can be done simply by manual counting without the necessity for any softwares and identity of the cell can be established by comparing with an already existing database.

CONCLUSIONS

The SCL-MCL PHA, Polyhydroxy propionate co-poly hydroxy ocatadecanoate co-polymer recovered from *Pseudomonas* sp. *MNNG-S* using a 14L scale fermenter, was solvent casted in pure form as well as in blends with other biodegradable polymers. The blending experiments were done in order to enhance the optical and processing properties of the polymer to make it suitable for cell culture. Among the blends PHA: PEG at 60:40 was most favored, as it was culture friendly with a superior rate of cellular proliferation. The biomaterial was found to be nontoxic and did not give rise to any malicious effect on the continuous cells, HeLa, HEp-2, Vero and McCoy. The electron microscopy of the scaffold showed that the solvent casted scaffold has a pore diameter of 40 μM which enabled cellular infiltration.

When extended to the limbal stem cell cultivation, this novel PHA based copolymer provided a supporting material to promote proliferation, differentiation and stratification of Corneal limbal epithelial stem cells. This polymer, like amniotic membrane, has the advantages of biocompatibility, biodegradability and biological inertness. Unlike amniotic membrane it is structurally uniform, more transparent, easy to handle, non biological, easy to manipulate to obtain the transparency and thickness by adjusting the blending ratio of the polymer with PEG.

On comparative use of commercially available polymers, Matrigel, mebiolgel and hydromatrix peptide hydrogel for cultivation of HSV-1, PHA: PEG showed a significantly good cellular proliferation with viral multiplication. PHA: PEG system would therefore be appropriate for viral multiplication with lesser immune response of the cells whereas, Hydromatrix peptide cell culture system seems to be efficient in eliciting cellular immune response on HSV-1 infection and would be appropriate for studies on host viral interaction.

To conclude, PHA based co polymer from indigenous species of *Pseudomonas* MNNG-S has a wide future in *in vitro* and *in vivo* applications of cell culture, as it gives, good viability and proliferation of continuous cultures, differentiating and stratifying capacity of primary corneal limbal stem cell culture and minimal innate immune response elicited in response to infection, in terms of TLRs studied.

LIMITATIONS AND FUTURE SCOPE OF THE STUDY

1. The study utilized solvent casted PHA polymer which gave porosity of 40 μm diameter which was good enough to support cellular infiltration and proliferation. But porosity of nanometer dimensions would have helped to give a better cellular micro environment. This study could not standardize an electro spinning method to achieve such nanodimensions. Nano dimensions would also have given a hypoxic environment to maintain the stemness of Corneal limbal epithelial stem cells for a longer duration. The study could be extended with an electrospinned scaffold to get a better support for 3d cell culture.

2. The study is purely *in vitro,* though *in vivo* studies would have given more information of the capability of the scaffold to be used as a carrier for limbal stem cell transplantation. Thus the study could be extended to evaluate further the possibilities of indigenous PHA based copolymer as a carrier for transplantation *in vivo* using an animal model

3. Only the response of HCE to HSV-1 was studied using different 3d systems. Hence, it did not give an adequate data to generalize the effect of host response to viral infections. The study would be extended further to evaluate host immune response with the infection of different viruses with different cell types in order to understand the effect of different 3d cultures.

SPECIFIC CONTRIBUTIONS

1. The study standardized DHPLC based STR fingerprinting of HEP-2 cell line with 3 STR loci namely vwa, DI6S539, D7S820 using HEp-2 cells for the first time in literature.
2. This study proved that indigenously produced polyhydroxyalkanoate, from *Pseudomonas MNNG-S*, based co- polymer (PHA: PEG, 60:40) is a cellular supportive biomaterial for continuous culture as it gave superior proliferation and viability compared to that of conventional culture plates.
3. This study gives an insight that the polymer would be an appropriate candidate for LSCT as it supported viability, proliferation, differentiation and stratification of primary corneal limbal cells with a good limbal marker expression on initial stages of culture and differentiated corneal epithelial marker expression at later stages of the culture.
4. The comparative study of the indigenous PHA based copolymer with commercially available gel based systems states that the polymer is efficient along with Hydromatrix peptide hydrogel, and matrigel in supporting cellular proliferation. The rate of proliferation of cells followed the order of Hydromatrix peptide hydrogel from Sigma, Indigenous PHA based copolymer and matrigel.
5. This study used indigenous PHA based copolymer for cultivation of HSV- 1 on HCE cell line and it reports to enhance viral proliferation with eliciting minimal immune response of the cells, in terms of TLRs tested. This study is novel as there is no literature available for the use of any membranous polymers for cultivation of viruses.

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APPENDIX

I. Method of trypsinization of cultured cells

- Media was drained from the cell culture flasks using a serological pipette, ensuring that the cells remain adherent to flask surface.
- The cells were rinsed with 1-10ml PBS depending on the size of the flask and appropriate volume of trypsin was added.
- The cell culture flask treated with trypsin was incubated at room temperature for 1-3 minutes.
- The flask was slightly tapped on the surface to dislodge the cells. It was confirmed by viewing under microscope that the cells are off the flask surface.
- Media was added to the flask (1-10 ml). The media was pipetted forcefully down the lower surface to dislodge the cells and to stop the trypsin.
- The cells were transferred to a pre-labeled test tube and centrifuged at 1000 rpm for 10 minutes.
- The supernatant was discarded carefully without disturbing the cell pellet and the pellet was re suspended in PBS.

II. Calculation of cell count by trypan blue exclusion method

Total number of viable cells per ml = number of viable cells counted × dilution

$$\frac{\text{Factor} \times \text{conversion factor (10 µl to 1ml}}{\text{Total area counted} \times \text{depth of the chamber}}$$

$$\boxed{\text{Total number of cells counted per square} \times 2 \times 10^4}$$

III. Extraction of DNA, by Quiagen DNA extraction kit method.

- A mixture of proteinase K, cell suspension and lysis buffer was prepared and placed in a water bath at 56 ^{0}C for 10 minutes.
- The contents were taken out from the water bath and transferred to a vial and ethanol was added to the contents of the vial. After thorough mixing, the vial was transferred to a column.

- The vial was placed in a centrifuge at 8000 rpm for 1 minute, following which the collection tube was discarded.
- Wash buffer I was added to the vial contents and the vial was once again placed in a centrifuge at 8000 rpm for 1 minute.
- Another round of centrifugation was performed at 14000 rpm for 3 minutes after addition of wash buffer II.
- The collection tube was discarded and the vial was subjected to an empty spin at 14000 rpm for 1 minute.
- The vial was discarded and the column was transferred to a sterile 1.5 ml vial.
- Elution buffer was added to the contents of the column and the vial containing the column was kept at room temperature for 1 minute.
- The vial was then centrifuged at 8000 rpm for 1 minute.
- The column was discarded and the vial was retained and stored in -20 ^{0}C refrigerator followed by quantification of the extracted DNA.

TABLE 7: Materials for DNA extraction

Component	Amount in µl
Proteinase K	20
HEp-2 cell suspension	200
Lysis buffer	200
Ethanol	200
Wash Buffer I	500
Wash Buffer II	500
Elution Buffer	200

IV. DNA quantification

NanoVue is a simple-to-use UV/Visible instrument with twin CCD array detectors (1024 pixels) and few moving parts, which contributes to its inherent reliability. The ratio of 260/280 was estimated along with DNA quantitation.

V. Agarose gel electrophoresis

- 2% Agarose gel was prepared by mixing 0.5 g of agarose powder in 50ml 1x buffer. The mixture was heated in oven, until agarose completely dissolves in the buffer.
- Once the mixture was taken out of the oven it was allowed to cool for a few minutes, after which 40 µl of ethidium bromide was added to it.
- The gel plate was readily prepared for pouring the gel mixture onto it. The combs were then placed in their positions in the gel plate and the gel was allowed to set.
- Once the gel was set, the combs were carefully removed without disturbing the wells formed by them.
- The gel plate was then placed in the electrophoresis apparatus. Adequate buffer was poured so as to immerse the gel to the required level.
- 10 µl of PCR product was mixed with 10 µl of gel loading dye and loaded on to the wells.
- The appropriate voltage was set in the electrophoresis apparatus and the products were allowed to run more three-forth across the gel.
- Once the run was over, the gel was viewed under UV spectrophotometer for bands and the gel picture was documented.
- PCR amplicons were loaded onto DHPLC for separation of STR alleles.

TABLE 8: Materials Required For Agarose Gel Electrophoresis

Components	Amount
Agarose powder	1 g
1X buffer	50 ml
Ethidium bromide	10 µl
PCR products	10 µl
Gel loading dye	10 µl

VI. Preparation of master mix for STR PCR:

TABLE 9: Master Reaction Mix for STR PCR

Component	Amount in µl
dNTPs (200 µM)	8
10x buffer	5
Forward primer	1
Reverse primer	1
MilliQ water	34.5
Taq Polymerase	0.5
Total	50

VII. Protocol for RNA Extraction.

- Scaffolds with limbal culture on day 2 and day 12 were stabilized with RNA later reagent.
- Stabilized scaffold was then placed in tissue grinder treated with DEPC and ground nicely with 50 µl buffer RLT provided in the kit.
- 350 µl of buffer RLT was added to the lysate and it was homogenized in the tissue grinder
- The lysate was then decanted to a 1 ml RNAase free vial and centrifuged at 10,000 rpm for 3 minutes.
- The supernatant was transferred to a new micro centrifuge tube and 1 volume of 70 % ethanol was added to the cleared lysate.
- Transferred up to 700 µl of the sample, including any precipitate that have formed, to an RNeasy spin column placed in a 2 ml collection tube supplied with the kit.
- The lid was closed gently and centrifuged for 15 s at 10,000 rpm, and the flow through was discarded.

- Added 700 µl Buffer RW1 to the RNeasy spin column, closed the lid gently, and centrifuged for 15 s at 10,000 rpm to wash the spin column membrane.
- Added 500 µl Buffer RPE to the RN easy spin column, closed the lid gently, and centrifuged for 15 s at 10,000 rpm to wash the spin column membrane.
- The flow through was discarded and the step was repeated again with centrifugation for 2 seconds
- In order to remove ethanol as long centrifugation dries up the membrane the spin column is collected in a new collection tube and centrifuged at 10,000 rpm for 1 minute.
- Placed the RNeasy spin column in a new 1.5 ml collection tube supplied with the kit and added 30–50 µl RNase-free water, directly to the spin column membrane.
- The lid was then closed gently, and centrifuged for 1 min at 10,000 rpm to elute the RNA.
- After quantitation the RNA was immediately converted to c DNA or stored at -80 ^{0}C.
- Integrity and quantity of the RNA was analyzed using Nanovue.

VIII. Conversion of RNA to cDNA

- The template RNA solution was thawed on ice.
- The oligo dT primers solutions, RNase inhibitor, 10x Buffer RT, dNTP Mix, and RNase-free water were brought at room temperature.
- Mixed each solution by vortexing, and centrifuged briefly to collect residual liquid from the sides of the tubes.
- Diluted RNase inhibitor (40 units/ µl) to a final concentration of 10 units/µl in ice-cold 1x Buffer RT.
- Mixed carefully by vortexing for no more than 5 s, and centrifuged briefly to collect residual liquid from the sides of the tube.

- Mastermix was prepared fresh on ice as per listed in appendix and mixed thoroughly and carefully by vortexing for no more than 5 s and centrifuged briefly to collect residual liquid from the walls of the tube, and store on ice.

- The template RNA was added to the individual tubes containing the master mix. Mixed thoroughly and carefully by vortexing for no more than 5 s and Centrifuged briefly to collect residual liquid from the walls of the tube.

- Incubated for 60 min at 37 ^{0}C

TABLE 10: Materials For cDNA Conversion

Component	Volume/ Reaction	Final concentration
10 X Buffer RT	2.0µl	1X
dNTP Mix(5mM each)	2.0 µl	0.5Mm each dNTP
Oligo-dT primer (10 µ M)	2.0 µl	1 µM
RNase inhibitor(10 U/ml)	1.0 µl	10 U/ml
Reverse transcriptase	1.0 µl	-
RNase free water	8.0 µl	-
Template RNA	4.0 µl	40 ng

LIST OF PUBLICATIONS

- **Shabna A**, Saranya V, Malathi J, Shenbagarathai R, Madhavan H.N. Indigenously produced polyhydroxyalkanoate based co-polymer as cellular supportive biomaterial. *Journal of biomedical materials research Part A*. 2014;102(10): 3470-6. Epub 2013/11/14.

- **Shabna.A**, Malathi J, Suman Kapur, Madhavan HN. Application of DHPLC based short tandem repeat profiling for authentication of human cell lines, Under review, *IJBT-68.*

- Narayanan Gomathy,Venil. N. Sumantran, **A.Shabna**, K. N. Sulochana. Tolerance of ARPE 19 cells to organophosphorus pesticide is limited to concentration and time of exposure. *Pestic Biochem Physiol*. 2015 Jan; 117:24-30. doi: 10.1016/j.pestbp.2014.10.004. Epub 2014 Oct 16.

- Saranya.V, **Shabna. A**, Malathi. J, Suman Kapur, Shenbagarathai. R, Madhavan H N.Indigenously produced polyhydroxyalkanoatebased co-polymer as a carrier for corneal limbal stem cell cultivation (Submitted BMM-100950).

- Shabna.A, Malathi.J, Suman Kapur, Shenbagarathai. R, Saranya. V, Madhavan H. N. A comparative study of HSV- 1 infectivity in matrix based HCE systems (UNDER PREPARATION).

LIST OF AWARDS

- **Swarnalatha Punshi Endowment Medal** for the best research work for the year 2013.
- **Senior Research Fellowship** from ICMR in the year 2013.
- **Travel grant** for oral presentation in IERG-ASIA ARVO- India chapter held at Hyderabad, 2011.
- **Travel fellowship** for ASIA ARVO, held at New Delhi, 2013
- **Wipro Biomed pvt limited Endowment Award** for biochemistry, 2010.
- Dr. S. Ramaswami & Dr.S.Narasimhan endowment award for human anatomy and human physiology, 2010.

BIOGRAPHY OF THE CANDIDATE

Ms. Shabna.A is a PhD student in Biological Sciences at Birla Institure of Technology and Sciences, Pilani. She received her three years Masters degree (MS) in MLT from BITS, Pilani in collaboration with Sankara Nethralaya chennai, and four years bachelors degree in MLT from Govt. Medical college Trivandrum under University of Kerala. Her thesis for post graduation involved The Effect of Chlorpyrifos (CP) On Human Retinal Pigment Epithelium - An *In Vitro* Study of Antioxidant Status And Apoptosis. She did her thesis work at the Department of Cell Biology, Sankara Nethralaya, Chennai. After joining L & T Microbiology Research Centre, Sankara Nethralaya in 2010 as a JRF in a DBT funded project, she registered for her PhD at BITS, PILANI under the supervision of Prof. H.N.Madhavan and Prof. Suman Kapur. In her thesis she aimed at **evaluating the scope of an indigenous polyhydroxy alkanoate based co-polymer in cell culture and virology**. Her works have been published in different peer reviewed scientific journals.

She has been pursuing the field of cell culture and virology and actively working in DBT funded project "Development of a PHA based polymer from *Pseudomonas LDC-5* and its mutant form *MNNG-S*. In 2013 she was awarded ICMR –SRF. She received Swarnalatha Punshi endowment medal for the best research work in the year 2013. She was awarded the Wipro Biomed pvt. limited endowment award for biochemistry and Dr. S. Ramaswami & Dr. S. Narasimhan endowment award for Human Anatomy and Human physiology in the year 2010. At present as an ICMR SRF she is working on the immune status of HSV-1 infected human corneal epithelial cells in different 3d cell culture systems.

BRIEF BIOGRAPHY OF THE CO-SUPERVISOR

Prof. Suman Kapur joined BITS, Pilani as Professor in the Centre for Biotechnology, Biological Sciences Group. She has worked in the capacity of Unit Chief, Community Welfare and International Relations since 1st January 2007. From 16th April 2010 she has taken charge as Dean, Research & Consultancy at the Hyderabad Campus of BITS. With her team of a dozen research scholars has been instrumental in building a state of the art Human Genomics laboratory from funds received as Principal and/or Co-Investigator of now more than eighteen grants awarded since her joining BITS in 2004. As a mentor she has been able to motivate younger faculty to submit and execute independent grants in the form Women scientist (DST), Research Associate and senior research fellows (ICMR & CSIR). She has published more than 80 research articles in International and journals. Her research interests life in identifying biomarkers for unraveling the genetic basis of human diseases such as psychianic disorders like depression, schizophrenia, addiction and alzheimeres disease and metabolic disorders such as diabetes (T2DM), obesity, cataract and metabolic syndrome. The group is specifically studying several genes,viz., *APOE, CAPN, PPARi5, it-4C ALDH2, ADM.% ADH1C, OPRM1, OB, TPH, CRVGA, CRVGB, D2, D5, ADCV4, ADCV3, CCKAR, CCKBR, cm, CF508, SPNK-1, PS-1, CVP2E1, CTSB, HSP70, TNFii, IC PRSS*-1 and several micro-satellite markers on chromosome segments 2, 6 and 10. Chronic diseases have a long latency period and genetic markers can be effectively used for identifying individuals at an increased risk for developing these diseases and advocating appropriate lifestyle measures to delay the onset and progression of such diseases. Prof. Suman Kapur has ably conducted the day to day activities of this unit and was instrumental in orchestrating several student exchanges introduction of new fellowships and opportunities for both students and faculty at BITS campuses.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Prof.H.N. Madhavan has completed his PhD degree in virology from Madras university in the year 1980. He received his MD Microbiology from Andhra University in the year l963 and MBBS from Madras university in the year 1957. As early as 1966 Dr. Madhavan established the tissue culture and virology laboratory in Jawaharlal Institute of Medical Education and Research and it was a WHO reporting centre for enterovirus diseases. His work on viral myocarditis has earned him an award of Ph.D. Degree by the University of Madras.He was a member of the Task force expert committee on enterovirus diseases of Indian Council of Medical Research during 1981.

Prof. Madhavan is known internationally for his research work in Medical Microbiology. He was among the earliest contributors on the investigations of enterovirus infections of the central nervous system in India. His report on the outbreak of Coxsackievirus B6 infection was the first in literature. His contribution on the association of Coxsackievirus B serotypes to human myocarditis is acclaimed as an outstanding piece of work. He was awarded Ph.D. by the University of Madras for his thesis on the role of viruses in myocarditis with particular reference to cell-mediated immunity in experimental Coxsackievirus B3 myocarditis.

Prof. Madhavan pioneered the development of diagnostic molecular microbiology as a specialized field in ocular infections. His department is the first in the country; to standardize molecular biological methods in the form of Polymerase chain reaction (PCR) based assays for the detection of infectious agents in ocular infections. He was the first to demonstrate the role of *M. tuberculosis* in the pathogenesis of Eales' disease by applying PCR to detect the presence the genome of this bacterium in the vitreous fluid and epiretinal membrane of these patients. PCR for detection of eubacterial genome and *Propionibacterium acnes* genome, PCR-based DNA probe hybridization to determine the Gram reaction of the infecting bacterium and detection of panfungal genome in intraocular fluids established by him have become the standard laboratory procedures in the investigation of infectious endophthalmitis. The PCRs for the detection of adenovirus and *Chlamydia trachomatis* in conjunctivitis developed by him are rapid diagnostic method. PCR methods developed by him to detect Herpes simplex virus, Varicella zoster virus and Cytomegalovirus in intraocular fluids by PCR are rapid, very sensitive and specific.

Using the molecular biological techniques developed and published in literature from his department, Dr. Madhavan along with other scientists in other institutions developed the World's First DNA Macro Chip which could simultaneously identify the infectious agent(s) in the clinicl specimens from ocular infections.

Since he found that the use of synthetic polymer, "Mebiol Gel" in collaboration with Prof Y. Mori of Waseda University Tokyo, Japan in cultivation of animal cell cultures is very promising, he has been studying its use in the cultivation of Corneal limbal epithelial stem cells. He has successfully cultivated Human Corneal Limbal Stem Tissue (HCLT) Cells obtained from eye donor tissues.

Dr.H.N.Madhavan has received Dr.U.C.Chaturvedi Lifetime Achievement award in the National Conference Microcons 2012 (Indian Association of Medical Microbiologists (IAMM) conference) at New Delhi. He has 5 patents and 213 publications. At present he is the director of L&T Microbiology Department,Vision Research Foundation, Sankara Nethralaya.