Theoretical course: Basic biochemical methods and ischemic heart models

Basics of Cell Culture

Anikó Görbe
Department of Biochemistry, Faculty of Medicine, University of Szeged
Cardiovascular Research Group
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Introduction

• Cell culture is the process by which prokaryotic, eukaryotic or plant cells are grown under controlled conditions
• In practice it refers to the culturing of cells derived from animal cells.
• Cell culture was first successfully undertaken by Ross Harrison in 1907
• Roux in 1885 for the first time maintained embryonic chick cells in a cell culture
Historical events in the development of cell culture

- 1878: Claude Bernard proposed that physiological systems of an organism can be maintained in a living system after the death of an organism.
- 1885: Roux maintained embryonic chick cells in a saline culture.
- 1897: Loeb demonstrated the survival of cells isolated from blood and connective tissue in serum and plasma.
- 1903: Jolly observed cell division of salamander leucocytes in vitro.
- 1907: Harrison cultivated frog nerve cells in a lymph clot held by the 'hanging drop' method and observed the growth of nerve fibers in vitro for several weeks. He was considered by some as the father of cell culture.
- 1910: Burrows succeeded in long term cultivation of chicken embryo cell in plasma clots. He made detailed observation of mitosis.
Continued...

- **1911**: Lewis and Lewis made the first liquid media consisted of sea water, serum, embryo extract, salts and peptones. They observed limited monolayer growth.

- **1913**: Carrel introduced strict aseptic techniques so that cells could be cultured for long periods.

- **1916**: Rous and Jones introduced proteolytic enzyme trypsin for the subculture of adherent cells.

- **1923**: Carrel and Baker developed 'Carrel' or T-flask as the first specifically designed cell culture vessel. They employed microscopic evaluation of cells in culture.

- **1927**: Carrel and Rivera produced the first viral vaccine - Vaccinia.

- **1933**: Gey developed the roller tube technique
Continued...

- 1940s: The use of the antibiotics penicillin and streptomycin in culture medium decreased the problem of contamination in cell culture.
- 1948: Earle isolated mouse L fibroblasts which formed clones from single cells. Fischer developed a chemically defined medium, CMRL 1066.
- 1952: Gey established a continuous cell line from a human cervical carcinoma known as HeLa (Helen Lane) cells. Dulbecco developed plaque assay for animal viruses using confluent monolayers of cultured cells.
- 1954: Abercrombie observed contact inhibition: motility of diploid cells in monolayer culture ceases when contact is made with adjacent cells.
- 1955: Eagle studied the nutrient requirements of selected cells in culture and established the first widely used chemically defined medium.
- 1961: Hayflick and Moorhead isolated human fibroblasts (WI-38) and showed that they have a finite lifespan in culture.
- 1964: Littlefield introduced the HAT medium for cell selection.
- 1965: Ham introduced the first serum-free medium which was able to support the growth of some cells.
Continued...

- 1965: Harris and Watkins were able to fuse human and mouse cells by the use of a virus.
- 1975: Kohler and Milstein produced the first hybridoma capable of secreting a monoclonal antibody.
- 1978: Sato established the basis for the development of serum-free media from cocktails of hormones and growth factors.
- 1982: Human insulin became the first recombinant protein to be licensed as a therapeutic agent.
- 1985: Human growth hormone produced from recombinant bacteria was accepted for therapeutic use.
- 1986: Lymphoblastoid γIFN licensed.
- 1987: Tissue-type plasminogen activator (tPA) from recombinant animal cells became commercially available.
- 1989: Recombinant erythropoietin in trial.
- 1990: Recombinant products in clinical trial (HBsAG, factor VIII, HIVgp120, CD4, GM-CSF, EGF, mAbs, IL-2).
Major development's in cell culture technology

- First development was the use of antibiotics which inhibits the growth of contaminants.
- Second was the use of trypsin to remove adherent cells to subculture further from the culture vessel.
- Third was the use of chemically defined culture medium.
Why is cell culture used for?

Areas where cell culture technology is currently playing a major role.

• **Model systems for**
  Studying basic cell biology, interactions between disease causing agents and cells, effects of drugs on cells, process and triggering of aging & nutritional studies

• **Toxicity testing**
  Study the effects of new drugs

• **Cancer research**
  Study the function of various chemicals, virus & radiation to convert normal cultured cells to cancerous cells
Why is cell culture used for?

- **Virology**
  
  Cultivation of virus for vaccine production, also used to study their infectious cycle.

- **Genetic Engineering**
  
  Production of commercial proteins, large scale production of viruses for use in vaccine production e.g. polio, rabies, chicken pox, hepatitis B & measles

- **Gene therapy**
  
  Cells having a functional gene can be replaced to cells which are having non-functional gene
Tissue culture

• In vitro cultivation of organs, tissues & cells at defined temperature using an incubator & supplemented with a medium containing cell nutrients & growth factors is collectively known as tissue culture.

• Different types of cell grown in culture includes connective tissue elements such as fibroblasts, skeletal tissue, cardiac, epithelial tissue (liver, breast, skin, kidney) and many different types of tumor cells.
Primary culture

- Cells when surgically or enzymatically removed from an organism and placed in suitable culture environment will attach and grow are called as primary culture
- Primary cells have a finite life span
- Primary culture contains a very heterogeneous population of cells
- Sub culturing of primary cells leads to the generation of cell lines
- Cell lines have limited life span, they passage several times before they become senescent
- Cells such as macrophages and neurons do not divide in vitro so can be used as primary cultures
- Lineage of cells originating from the primary culture is called a cell strain
Making a Primary Culture

1. Remove tissue
2. Mince or chop
3. Digest with proteolytic enzymes
4. Place in culture

Enzymatic Dissociation
PRIMARY CARDIOMYOCYTE CULTURE

Animal:

• newborn rats (Wistar)

Heart isolation:

• hearts were excised and collected in sterile PBS

Digestion:

• after washing in fresh PBS, atria were removed and ventricules were cut into small pieces and put into the trypsin (0.25%) solution
• digestion was proceed in 37 degree in the water bath for 25 min and the cell suspension was resuspended every 5 minutes using 5 ml pipettes
PRIMARY CARDIOMYOCYTE CULTURE

Washing and cell collection:

- Centrifuge the cell suspension in 2000 rpm for 15 min
- Remove the supernatant and resuspend the cells in growing medium (DMEM + 10% fetal calf serum (FCS) + gentamicin) – high glucose and glutamine content in the medium

Preplating: fibroblast elimination

- Plate the cells into 6-well plates for 30 min
- Collect the supernatant (non-attached cells) into a Falcon tube

Plating:

- 6-well plate we plate $5 \times 10^5$ cells/ well
- Keep them at 37 and 5% CO$_2$ and change the medium next day (24h)
<table>
<thead>
<tr>
<th></th>
<th><strong>NEONATAL</strong></th>
<th><strong>ADULT</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>animal</td>
<td>Newborn- cheap (limited cell number can be achieved)</td>
<td>Adult –expensive, but more cells</td>
</tr>
<tr>
<td>preparation</td>
<td>simple</td>
<td>more complicated</td>
</tr>
<tr>
<td>sensitivity</td>
<td>relatively easy to handle</td>
<td>very sensitive</td>
</tr>
<tr>
<td>life time</td>
<td>couple of days, max 2 weeks (long term experiments as well)</td>
<td>max 2-3 days (only short term experiments)</td>
</tr>
<tr>
<td>contamination</td>
<td>yes, fibroblasts</td>
<td>not observed</td>
</tr>
<tr>
<td>coated surface</td>
<td>not required</td>
<td>exclusively laminin coated surface can be used</td>
</tr>
<tr>
<td>medium</td>
<td>DMEM</td>
<td>Medium 199 + additional comp</td>
</tr>
<tr>
<td>identification</td>
<td>IH of cardiomyocytes</td>
<td>microscopic morphology</td>
</tr>
<tr>
<td>adherent</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>
2 days old neonatal cardiac myocyte culture
1 day old adult cardiac myocyte culture
Majority Of Cells Adhere On Plastic (Treated) Provided They Are Not Transformed

It Was Observed That Cells Prefer –vely Charged Glass Surface

Plastic (polystyrene) Is Tissue Culture Treated
  • With High Energy Ionizing Radiation
  • Electric Ion Discharge

Adhesion Is Mediated By Surface Receptors And Matrix
  • Matrix Is Secreted By Cells, Adheres To Charged Plastic
  • Receptors Bind to Matrix
Three Major Classes

- **Cell-Cell Adhesion Molecules**
  - CAMs (Ca$^{2+}$ Independent)
  - Cadherins (Ca$^{2+}$ Dependent)
  - Primarily Between Homologous Cells
  - Signaling occurs

- **Cell-Substrate Molecules**
  - Integrins
  - Bind to fibronectin, entactin, laminin, collagen
  - Bind the specific motif (RGD, arginine, glycine, aspartic)
  - Comprised of $\alpha$ and $\beta$ unit
Cell Surface Adhesion Molecules

- 3rd Class Is Proteoglycans
  - Also Binds Matrix or Other Proteoglycans
  - Not Via RGD Motif
  - Low affinity Growth Factor Receptors
  - May Aid Binding To Higher Affinity Receptors
  - No Signaling Capacity
Cell Lines

Cell Line

- Cells that have undergone a mutation and won’t undergo apoptosis after a limited number of passages. They will grow indefinitely.

Transformed/continuous cell line

- A cell line that has been transformed by a tumor inducing virus or chemical. Can cause tumors if injected into animal.

Hybrid cell line (hybridoma)

- Two cell types fused together with characteristics of each
Continous cell lines

- Most cell lines grow for a limited number of generations after which they ceases
- Cell lines which either occur spontaneously or induced virally or chemically transformed into Continous cell lines

Characteristics of continous cell lines
- smaller, more rounded, less adherent with a higher nucleus /cytoplasm ratio
- fast growth and have aneuploid chromosome number
- reduced serum and anchorage dependence and grow more in suspension conditions
- ability to grow upto higher cell density
- different in phenotypes from donar tissue
- stop expressing tissue specific genes
Common cell lines

Human cell lines
- MCF-7: breast cancer
- HL 60: Leukemia
- HEK-293: Human embryonic kidney
- HeLa: Henrietta lacks

Primate cell lines
- Vero: African green monkey kidney epithelial cells
- Cos-7: African green monkey kidney cells

And others such as CHO from hamster, sf9 & sf21 from insect cells
Types of cells

On the basis of morphology (shape & appearance) or on their functional characteristics. They are divided into three groups:

• **Epithelial like**- attached to a substrate and appears flattened and polygonal in shape
• **Lymphoblast like**- cells do not attach remain in suspension with a spherical shape
• **Fibroblast like**- cells attached to an substrate appears elongated and bipolar
Culture media

- Choice of media depends on the type of cell being cultured
- Commonly used Medium are GMEM, EMEM, DMEM etc.
- Media is supplemented with antibiotics viz. penicillin, streptomycin etc.
- Prepared media is filtered and incubated at 4°C
Culturing of cells

- Cells are cultured as anchorage dependent or independent
- Cell lines derived from normal tissues are considered as anchorage-dependent grows only on a suitable substrate e.g. tissue cells
- Suspension cells are anchorage-independent e.g. blood cells
- Transformed cell lines either grows as monolayer or as suspension
Why sub culturing?

- Once the available substrate surface is covered by cells (a confluent culture) growth slows & ceases.
- Cells to be kept in healthy & in growing state have to be sub-cultured or passaged.
- It’s the passage of cells when they reach to 80-90% confluency in flask/dishes/plates.
- Enzyme such as trypsin, collagenase in combination with EDTA breaks the cellular glue that attached the cells to the surface.
Passage of Adherent cells

- Cells which are anchorage dependent
- Cells are washed with PBS (free of Ca & Mg) solution.
- Add enough trypsin/EDTA to cover the monolayer
- Incubate the plate at 37 C for 1-2 mts
- Tap the vessel from the sides to dislodge the cells
- Add complete medium to dissociate and dislodge the cells
- with the help of pipette which are remained to be adherent
- Add complete medium depends on the subculture requirement either to 75 cm or 175 cm flask.
Suspension cells

- Easier to passage as no need to detach them
- As the suspension cells reach to confluency
- Asceptically remove 1/3\textsuperscript{rd} of medium
- Replaced with the same amount of pre-warmed medium
Working with cryopreserved cells

- Vial from liquid nitrogen is placed into 37°C water bath, agitate vial continuously until medium is thawed
- Centrifuge the vial for 10 mts at 1000 rpm at RT, wipe top of vial with 70% ethanol and discard the supernatant
- Resuspend the cell pellet in 1 ml of complete medium with 20% FBS and transfer to properly labeled culture plate containing the appropriate amount of medium
- Check the cultures after 24 hrs to ensure that they are attached to the plate
- Change medium as the colour changes, FBS until the cells are established
Freezing cells for storage

- Remove the growth medium, wash the cells by PBS and remove the PBS by aspiration
- Dislodge the cells by trypsin-verseene
- Dilute the cells with growth medium
- Transfer the cell suspension to a 15 ml conical tube, centrifuge at 200g for 5 mts at RT and remove the growth medium by aspiration
- Resuspend the cells in 1-2ml of freezing medium
- Transfer the cells to cryovials, incubate the cryovials at -80 C overnight
- Next day transfer the cryovials to Liquid nitrogen
Confluence

• How “covered” the growing surface appears
• This is usually a guess
• Optimal confluency for moving cells to a new dish is 70-80%
  • too low, cells will be in lag phase and won’t proliferate
  • Too high and cells may undergo unfavorable changes and will be difficult to remove from plate.
Cell toxicity

- Cytotoxicity causes inhibition of cell growth
- Observed effect on the morphological alteration in the cell layer or cell shape
- Characteristics of abnormal morphology is the giant cells, multinucleated cells, a granular bumpy appearance, vacuoles in the cytoplasm or nucleus
- Cytotoxicity is determined by substituting materials such as medium, serum, supplements flasks etc. at a time
## Viability assay – DEAD CELLS

<table>
<thead>
<tr>
<th></th>
<th>Trypane blue exclusion</th>
<th>Propidium iodine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biological indicator</strong></td>
<td>Membrane integrity</td>
<td>Membrane integrity</td>
</tr>
<tr>
<td><strong>Detection method</strong></td>
<td>Colorimetric microscopy</td>
<td>Fluorescent FACS</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td>2 h</td>
<td>30 min</td>
</tr>
<tr>
<td><strong>limitation</strong></td>
<td>24 groups max</td>
<td>$10^6$ cells/well required + Imre</td>
</tr>
</tbody>
</table>
# Viability assay – LIVING CELLS

<table>
<thead>
<tr>
<th>Biological indicator</th>
<th>Cell-Titer Glo (ATP)</th>
<th>Cell Titer Blue resazurin</th>
<th>Cell Titer 96 (MTS)</th>
<th>MTT</th>
<th>LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP content</td>
<td>ATP content</td>
<td>Reducing ability</td>
<td>Reducing ability</td>
<td>Reducing ability</td>
<td>Membrane integrity</td>
</tr>
<tr>
<td>Detection method</td>
<td>bioluminescent</td>
<td>Fluorimetric or colorimetric</td>
<td>colorimetric</td>
<td>colorimetric</td>
<td>UV</td>
</tr>
<tr>
<td>Time</td>
<td>10 min</td>
<td>1-4 h</td>
<td>1-4 h</td>
<td>4 h</td>
<td>10 min</td>
</tr>
<tr>
<td>Sensitivity (96 plate)</td>
<td>50 cells</td>
<td>390 cells</td>
<td>800 cells</td>
<td>800 cells</td>
<td>800 cells</td>
</tr>
<tr>
<td>Limitation</td>
<td>Lumi reader</td>
<td>Fluoro reader</td>
<td></td>
<td></td>
<td>UV reader</td>
</tr>
</tbody>
</table>
Cell viability

- Cell viability is determined by staining the cells with trypan blue.
- As trypan blue dye is permeable to non-viable cells or death cells whereas it is impermeable to this dye.
- Stain the cells with trypan dye and load to haemocytometer and calculate % of viable cells:
  \[
  \% \text{ of viable cells} = \frac{\text{Nu. of unstained cells}}{\text{total nu. of cells}} \times 100
  \]
Hemacytometer

- Specialized chamber with etched grid used to count the number of cells in a sample.
- Use of trypan blue allows differentiation between living and dead cells.
Using the Hemacytometer

- Take the hemacytometer and coverslip (carefully) and dry thoroughly with a kimwipe.
- Center coverslip on hemacytometer
- Barely fill the grid under the coverslip via the divet with your cell suspension.
- Count cells in ten squares (5 on each side) by following diagram at station.
Looking at the grid under the phase contrast microscope
How the cells will appear

• Bright refractile “spheres” are living cells,

• Blue cells about the same size as the other cells are dead.

• Keep a differential count of blue vs. clear for viability determination.

• Sometimes there will be serum debris, and this will look red or blue and stringy or gloppy--don’t count it!

These are blood cells, You will not have this many
Viability assay – PI fluorescence intensity determined by plate reader

Propidium iodine

Cardiac myocytes

www.cardiovasc.com
SIMULATED ISCHEMIA in HYPOXIC CHAMBER

95% N₂ and 5% CO₂ gas
## CONTROL SOLUTION

<table>
<thead>
<tr>
<th></th>
<th>Molecular weight</th>
<th>mM</th>
<th>For 1 L (g)</th>
<th>For 100 ml (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>58.44</td>
<td>125</td>
<td>7,305</td>
<td>730</td>
</tr>
<tr>
<td>KCl</td>
<td>74.56</td>
<td>5.4</td>
<td>0,402</td>
<td>40.2</td>
</tr>
<tr>
<td>NaH₂PO₄·1 H₂O</td>
<td>137,99</td>
<td>1.2</td>
<td>0,1655</td>
<td>16.6</td>
</tr>
<tr>
<td>MgCl₂ (saturated solution)</td>
<td>95.23</td>
<td>0.5</td>
<td>126 ul</td>
<td>12.6 ul</td>
</tr>
<tr>
<td>HEPES</td>
<td>238.3</td>
<td>20</td>
<td>4,766</td>
<td>476.6</td>
</tr>
<tr>
<td>glucose</td>
<td>198.18</td>
<td>15</td>
<td>2,972</td>
<td>297.2</td>
</tr>
<tr>
<td>taurine</td>
<td>125.1</td>
<td>5</td>
<td>0.6255</td>
<td>62.6</td>
</tr>
<tr>
<td>CaCl₂·2 H₂O</td>
<td>174.02</td>
<td>1</td>
<td>0.174</td>
<td>17.4</td>
</tr>
<tr>
<td>(Creatine P·Na₂·6 H₂O)</td>
<td>363.2</td>
<td>2.5</td>
<td>0.908</td>
<td>90.8</td>
</tr>
<tr>
<td>creatine</td>
<td>131.14</td>
<td>2.5</td>
<td>0.328</td>
<td>32.79</td>
</tr>
<tr>
<td>BSA</td>
<td>0.1%</td>
<td></td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

pH: set to 7.4 (pH 6 was after mixing components)

MgCl₂ (saturated solution): 1 ml contains 0.3774 g

## HYPOXIC SOLUTION

<table>
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<tr>
<th></th>
<th>Molecular weight</th>
<th>mM</th>
<th>For 1 L (g)</th>
<th>For 100 ml (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>58.44</td>
<td>119</td>
<td>6,954</td>
<td>695.4</td>
</tr>
<tr>
<td>KCl</td>
<td>74.56</td>
<td>5.4</td>
<td>0,402</td>
<td>40.2</td>
</tr>
<tr>
<td>NaH₂PO₄·1 H₂O</td>
<td>137,99</td>
<td>1.2</td>
<td>0,1655</td>
<td>16.6</td>
</tr>
<tr>
<td>MgCl₂ (saturated solution)</td>
<td>95.23</td>
<td>0.5</td>
<td>126 ul</td>
<td>12.6 ul</td>
</tr>
<tr>
<td>HEPES</td>
<td>238.3</td>
<td>5</td>
<td>1,1915</td>
<td>119.2</td>
</tr>
<tr>
<td>MgSO₄·7 H₂O</td>
<td>246.48</td>
<td>1.3</td>
<td>0.320</td>
<td>32</td>
</tr>
<tr>
<td>CaCl₂·2 H₂O</td>
<td>174.02</td>
<td>0.9</td>
<td>0.1566</td>
<td>15.7</td>
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<tr>
<td>Na-lactate</td>
<td>112.1</td>
<td>20</td>
<td>2,242</td>
<td>224</td>
</tr>
<tr>
<td>BSA</td>
<td>0.1%</td>
<td></td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

pH: set to 6.4 (pH 6.4 was after mixing components)

MgCl₂ (saturated solution): 1 ml contains 0.3774 g
Contaminant’s of cell culture

Cell culture contaminants of two types

- **Chemical**-difficult to detect caused by endotoxins, plasticizers, metal ions or traces of disinfectants that are invisible
- **Biological**-cause visible effects on the culture they are mycoplasma, yeast, bacteria or fungus or also from cross-contamination of cells from other cell lines
Effects of Biological Contamination’s

- They competes for nutrients with host cells
- Secreted acidic or alkaline by-products ceases the growth of the host cells
- Degraded arginine & purine inhibits the synthesis of histone and nucleic acid
- They also produces $\text{H}_2\text{O}_2$ which is directly toxic to cells
Detection of contaminants

- In general indicators of contamination are turbid culture media, change in growth rates, abnormally high pH, poor attachment, multi-nucleated cells, graining cellular appearance, vacuolization, inclusion bodies and cell lysis.
- Yeast, bacteria & fungi usually shows visible effect on the culture (changes in medium turbidity or pH).
- Mycoplasma detected by direct DNA staining with intercalating fluorescent substances e.g. Hoechst 33258.
- Mycoplasma also detected by enzyme immunoassay by specific antisera or monoclonal abs or by PCR amplification of mycoplasmal RNA.
- The best and the oldest way to eliminate contamination is to discard the infected cell lines directly.
Basic equipments used in cell culture

• **Laminar cabinet**- Vertical are preferable
• **Incubation facilities**- Temperature of 25-30 C for insect & 37 C for mammalian cells, co2 2-5% & 95% air at 99% relative humidity. To prevent cell death incubators set to cut out at approx. 38.5 C
• **Refrigerators**- Liquid media kept at 4 C, enzymes (e.g. trypsin) & media components (e.g. glutamine & serum) at -20 C
• **Microscope**- An inverted microscope with 10x to 100x magnification
• **Tissue culture ware**- Culture plastic ware treated by polystyrene
**CO₂ incubator**

- maintains CO₂ level (5-10%), humidity and temperature (37°C) to simulate in vivo conditions.
Water bath

- To warm media and PBS before placing on cells
- Can harbor fungi and bacteria, spray all items with 70% ethanol before placing in the hood.
- Usually takes 10-15 minutes for media to warm
Inverted Phase Microscope

- A phase contrast microscope with objectives *below* the specimen.
- A phase plate with an annulus will aid in exploiting differences in refractive indices in different areas of the cells and surrounding areas, creating contrast.
Mechanics of phase microscopy

Shifting of phase by \( \frac{1}{2} \) a wavelength
Add and subtract amplitudes to create more contrast
A comparison

Phase contrast microscopy
Can be used on living cells

Light microscopy
requires stain, thus killing cells
Basic aseptic conditions

- If working on the bench use a Bunsen flame to heat the air surrounding the Bunsen
- Swab all bottle tops & necks with 70% ethanol
- Flame all bottle necks & pipette by passing very quickly through the hottest part of the flame
- Avoiding placing caps & pipettes down on the bench; practice holding bottle tops with the little finger
- Work either left to right or vice versa, so that all material goes to one side, once finished
- Clean up spills immediately & always leave the work place neat & tidy
Safety aspect in cell culture

• Possibly keep cultures free of antibiotics in order to be able to recognize the contamination
• Never use the same media bottle for different cell lines. If caps are dropped or bottles touched unconditionally touched, replace them with new ones
• Necks of glass bottles prefer heat at least for 60 secs at a temperature of 200 C
• Switch on the laminar flow cabinet 20 mts prior to start working
• Cell cultures which are frequently used should be subcultered & stored as duplicate strains
Reference:

Paras Yadav\textsuperscript{1}, Annu Yadav\textsuperscript{1}, P. Kumar\textsuperscript{1}, J.S. Arora\textsuperscript{1}, T.K.Datta\textsuperscript{1}, S. De\textsuperscript{1}, S.L. Goswami\textsuperscript{1}, Mukesh Yadav\textsuperscript{2}, Shalini Jain\textsuperscript{3}, Ravinder Nagpal\textsuperscript{4} and Hariom Yadav\textsuperscript{3}

\textsuperscript{1}Department of Animal Biotechnology, \textsuperscript{2}Animal Biochemistry Division and \textsuperscript{4}Dairy Microbiology Division, National Dairy Research Institute, Karnal 132001 (Haryana), India; \textsuperscript{2}SOS in Chemistry, Jiwaji University, Gwalior-474011, M.P., India