Code: AS-2248

M.Sc. III Semester Biotechnology
End Semester Examination, 2013
LBTM: 304 Animal Biotechnology

Model Answers

Answer Key for Section-A (Objective Type Questions)

Question 1.

(i) (a) Inverted microscope
(ii) (b) Contact inhibition
(iii) (c) Both of the above
(iv) (d) Both a and c
(v) (d) Cells are dissociated enzymatically
(vi) (d) All of the above
(vii) (d) All of the above
(viii) (b) One copy of the original stem cell and a second non-stem cell
(ix) (b) Reversible
(x) (a) Milk

Model Answers for Section-B (Long Answer Type Questions)

Answer 2: A tissue culture laboratory should be designed with the following considerations:

(i) Layout of aseptic room:
- (a) Sterile handling area: Sterile work should be located in a quiet part of the tissue culture laboratory and should be restricted to tissue culture work only and there should be no traffic or other disturbances.
- (b) Laminar Flow: The use of laminar-flow hoods with sterile air blown onto the work surface affords greater control of sterility at a lower cost than providing a separate sterile room.
- (c) Quarantine and Containment: Newly imported cell lines or biopsies can be handled in a separate aseptic room with its own laminar-flow hood, incubators, freezer, refrigerator, centrifuge, supplies, and disposal until they are shown to be free of contamination, particularly mycoplasma.
- (d) Service Bench: The service bench should be placed for a cell counter, microscope, etc., close to the sterile handling area and should also provide for the storage of sterile glassware, plastics, pipettes, screw caps, syringes, etc., in drawer units below and open shelves above.

(ii) Incubation: Incubation may be carried out in separate incubators or in a thermostatically controlled hot room. The temperature of the hot room should be controlled within ±0.5°C at any point and at any time and depends on the sensitivity and accuracy of the control assembly, the location of the thermostat sensor, the circulation of air in the room, the nature of the insulation, and the evolution of heat by other apparatus in the room.

(iii) Preparation Area:

(a) Media Preparation: If reliable commercial media is not available or a laboratory needs to prepare its own media, the preparation area should be large enough to accommodate a coarse and a fine balance, a pH meter, and an osmometer. Bench space will be required for dissolving and stirring solutions and for bottling and packaging various materials, and additional ambient and refrigerated shelf space will also be needed. If possible, an extra horizontal laminar-flow hood should be provided in the sterile area for filtering and bottling sterile liquids, and incubator space must be allocated for quality control of sterility. Heat-stable solutions and equipment can be autoclaved or dry-heat sterilized.

(b) Washup: Washup and sterilization facilities should be placed outside the tissue culture lab, as the humidity and heat that they produce may be difficult to dissipate without increasing the airflow above desirable limits. Autoclaves, ovens, and distillation apparatus should be located in a separate room with an efficient extraction fan.

(c) Storage: The different components should be stored as: Liquids: ambient, 4°C, −20°C; Glassware (shelving); Plastics (shelving); Small items (drawers); Specialized equipment (slow turnover), cupboard(s); Chemicals: ambient, 4°C, −20°C; CO_2 cylinders; Space for liquid N_2 freezer(s).

(iv) Useful additions: Piped CO_2 and compressed air, storeroom for bulk plastics, quarantine room, containment room, Liquid N_2 storage tank (~500 L) and separate storeroom for nitrogen freezers, microscope room, darkroom and vacuum line.

Answer 3: Following are the advantages and disadvantages of serum free media:

Advantages:

1. Serum free media does not have the disadvantages of serum such as physiological variability, shelf life and consistency, quality control, specificity, availability, downstream processing, contamination, cost, growth inhibitors and standardization.
2. Selective Media: Serum-free media provides control over growth promoting activity which provides the ability to be selective for a particular cell type. For example, fibroblastic overgrowth can be inhibited in breast and skin cultures by using MCDB 170 and 153 and melanocytes can be cultivated in the absence of fibroblasts and keratinocytes.
3. Regulation of Proliferation and Differentiation: Serum-free media can be switched from a growth-enhancing medium for propagation to a differentiation-inducing medium by altering the concentration and types of growth factors and other inducers.

Disadvantages:

1. Multiplicity of Media: Each cell type requires a different recipe of media, and cultures from malignant tumors may vary in requirements from tumor to tumor. This presents a problem for laboratories maintaining cell lines of several different origins as they need several different types of media.
2. Selectivity: Some media may select a sublineage that is not typical of the whole population. Cells at different stages of development (e.g., stem cells vs. committed precursor cells) may require different formulations, particularly in the growth factor and cytokine components.

3. Reagent Purity: The removal of serum also requires that the degree of purity of reagents and water and the degree of cleanliness of all apparatus be extremely high, as the removal of serum also removes the protective, detoxifying action that some serum proteins may have.
4. Cell Proliferation: Growth is often slower in serum free media, and fewer generations are achieved with finite cell lines.
5. Availability: The availability of properly quality-controlled serum-free media is quite limited, and the products are often more expensive than conventional media.

Answer 4: Using serum in a medium has the following disadvantages:

(i) Physiological Variability: The major constituents of serum, such as albumin and transferrin, are known, but the concentrations and actions of minor components such as nutrients (amino acids, nucleosides, sugars, etc.), peptide growth factors, hormones, minerals, and lipids have not been fully determined.

(ii) Shelf Life and Consistency: Serum has a low shelf-life and also varies from batch to batch. A batch will last for one year under ideal conditions, perhaps deteriorating during that time. It must then be replaced with another batch that may be selected as similar, but will never be identical, to the first batch.

(iii) Quality Control: Strict quality control is needed during changing of serum batches to ensure that the replacement is as close as possible to the previous batch. This can involve several tests (for growth, plating efficiency, and special function) and a number of different cell lines.

(iv) Specificity: A different batch of serum is needed for more than one cell type, so that several batches must be held on reserve simultaneously.

(v) Availability: Sometimes, the supply of serum is restricted because of drought in the cattle-rearing areas, the spread of disease among the cattle, or economic or political reasons. This can create problems at any time, restricting the amount of serum available and also the number of batches to choose from.

(vi) Downstream Processing: The presence of serum creates a major obstacle to product purification and may even limit the pharmaceutical acceptance of the product.

(vii) Contamination: Serum is frequently contaminated with viruses, most of which may be harmless to cell culture but represent an additional unknown factor outside the operator's control and which may influence the composition and properties of the media.

(viii) Cost: Cost is often considered as a disadvantage of serum supplementation as serum constitutes the major part of the cost of a bottle of medium (more than 10 times the cost of the chemical constituents).

(ix) Growth Inhibitors: The net effect of the serum is an unpredictable combination of both inhibition and stimulation of growth. Stimulation usually predominates, although substances such as hydrocortisone is cytostatic to many cell types, such as glia and lung epithelium and TGF-β, released from platelets, is cytostatic to many epithelial cells.

(x) Standardization: Standardization of experimental and production protocols is difficult, both at different times and among different laboratories, because of batch-to batch variations in serum.

Answer 5: Cell separation refers to the ability to sort cells into distinct populations for the study of individual cell types isolated from a heterogeneous starting population without contamination from other cell types.

Various techniques for cell separation are as follows:

1. **Cell density and Isopyknic sedimentation:** The cells sediment in a density gradient to an equilibrium position equivalent to their own density. The density medium should be nontoxic and nonviscous at high densities (1.10 g/mL) and should exert little osmotic pressure in solution.
2. **Cell size and sedimentation velocity:**
   The relationship between the particle size and sedimentation rate at 1 *g* can be expressed approximately as $v \approx r^2/4$, where *v* is the sedimentation rate in mm/h and *r* is the radius of the cell in μm
   (a) Unit Gravity Sedimentation: Layering cells over a serum gradient in medium will allow the cells to settle through the medium according to size. The drawbacks are that it doesn't work with large number of cells and works with major differences in cell size or where aggregates are separated from single cells.
   (b) Centrifugal Elutriation: The centrifugal elutriator (Beckman Coulter) is a device for increasing the sedimentation rate and improving the yield and resolution of cell separation by performing the separation in a specially designed centrifuge and rotor. Cells in the suspending medium are pumped into the separation chamber in the rotor while it is turning. While the cells are in the chamber, centrifugal force tends to push the cells to the outer edge of the rotor. Meanwhile, medium is pumped through the chamber such that the centripetal flow rate balances the sedimentation rate of the cells. Cells of differing sedimentation rates will therefore reach equilibrium at different positions in the chamber. When the cells are seen to reach equilibrium, the flow rate is increased and the cells are pumped out into receiving vessels.
3. **Fluorescence-Activated Cell Sorting (FACS)**
   FACS operates by projecting a single stream of cells through a laser beam in such a way that the light scattered from the cells is detected by one or more photomultipliers and recorded. A fluorescence-activated cell sorter (FACS) is an instrument that uses the emission signals from each cell to sort the cell into one of two sample collection tubes and a waste reservoir. Cell stream in D-PBSA enters at the top, and sheath liquid, also D-PBSA, is injected around the cell stream to generate a laminar flow within the flow chamber. When the cell stream exits the chamber, it cuts a laser beam, and the signal generated triggers the charging electrode, thereby charging the cell stream. The cell stream then breaks up into droplets, induced by the 15-kHz vibration transducer attached to the flow chamber. The droplets carry the charge briefly applied to the exiting cell stream and are deflected by the electrode plates below the flow chamber.
4. **Antibody-based techniques**
   (a) **Immune Panning**: A cell-type-specific antibody raised against a cell surface epitope is conjugated to the bottom of a Petri dish, and when the mixed cell population is added to the dish, the cells to which the antibody is directed attach rapidly to the bottom of the dish. The remainder can then be removed.
   (b) **Magnetic Sorting**: Magnetic sorting uses a specific antibody, raised against a cell surface epitope, conjugated to ferritin beads or microbeads. When the cell suspension is mixed with the beads and then placed in a magnetic field, the cells that have attached to ferritin beads are drawn to the side of the

separating chamber. The cells and beads are released when the current is switched off, and the cells may be separated from the beads by trypsinization or vigorous pipetting.

Answer 6: After the first subculture, or passage, the primary culture becomes known as a cell line and may be propagated and subcultured several times.

(i) Differences between finite and continuous cell lines

S.No	Property	Finite cell Line	Continuous cell line
1	Ploidy	Euploid, diploid	Aneuploid, heteroploid
2	Transformation	Normal	Immortal, growth control altered, and tumorigenic
3	Anchorage dependence	Yes	No
4	Contact Inhibition	Yes	No
5	Mode of growth	Monolayer	Monolayer or Suspension
6	Serum requirement	High	Low
7	Density limitation of cell proliferation	Yes	Reduce or lost
8	Growth Rate	Slow	Rapid
9	Markers	Tissue specific	Chromosomal, enzymic, antigenic
10	Senescence	Yes	No

(ii) Differences between Adherent and Suspension Culture

S.No	Adherent Culture	Suspension Culture
1	Cells attach to surface	Free floating in culture medium
2	Anchorage dependence	Anchorage independence
3	Appropriate for most cell types	Appropriate for hematopoietic cells, transformed cell lines and cells from malignant tumours
4	Requires periodic passaging	Easier to passage, requires daily cell counts and viability determination
5	Cells are dissociated enzymatically or mechanically	No need for enzymatic dissociation of cells
6	Growth is limited by surface area	Growth is limited by concentration of cells in the media
7	Requires tissue culture treated vessel	Does not need tissue culture treated vessel
8	Used for cytology, harvesting products continuously and research applications	Used for bulk protein production, batch harvesting and research applications
9	Does not need agitation	Requires agitation for adequate gas exchange
10	Easy visual inspection under inverted microscope	No visual inspection possible

Answer 7: Viral vaccines production:

(a) Hepatitis B: Recombinant hepatitis B vaccine as a subunit vaccine is produced by cloning HBsAg gene in yeast cells. The gene for HBsAg is inserted (pMA 56) which is linked to the alcohol dehydrogenase promoter. These plasmids are then transferred and cultured. The cells grown in tryptone free medium are selected and cloned. The yeast cells are cultured. The HBsAg gene is expressed to produce 2nm sized particles similar to those found in patients infected with hepatitis B. These particles are immunoreactive with anti-HBsAg antibodies.

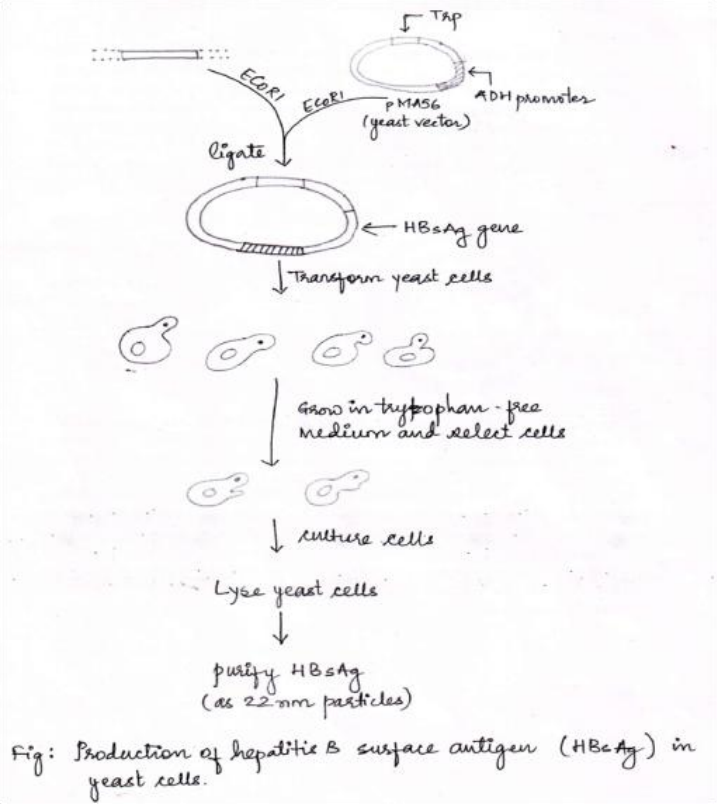


Fig: Production of hepatitis B surface antigen (HBsAg) in yeast cells.

(b) Foot and Mouth Disease (FMD): The genome of FMD is composed of single stranded RNA, covered by four viral proteins (VP1, VP2, VP3 and VP4). Among these, VP1 is immunogenic. The nucleotide sequence encoding VP1 was identified in the FMDV genome. A double stranded cDNA from the single stranded viral RNA was synthesized. This cDNA was then digested with restriction enzymes and the fragments were then cloned by using plasmid pBR322 in *E. coli.* The recombinant vaccine for FMDV in the form of viral protein 1 was used to vaccinate animals. Peptide vaccines for FMD were also prepared by chemically synthesizing the domains of VP1 and FMDV.

(c) Herpes Simplex Virus (HSV): An envelope glycoprotein D (gD) of HSV that can elicit antibody production has been identified. This is a membrane bound protein and difficult to isolate and purify. The glycoprotein D was modified by deleting the transmembrane portion of the protein and the gene was modified. This gene for gD was cloned in a mammalian vector and expressed in Chinese Hamster Ovary (CHO). In the experimental trials, the modified form of gD was found to be effective against HSV.

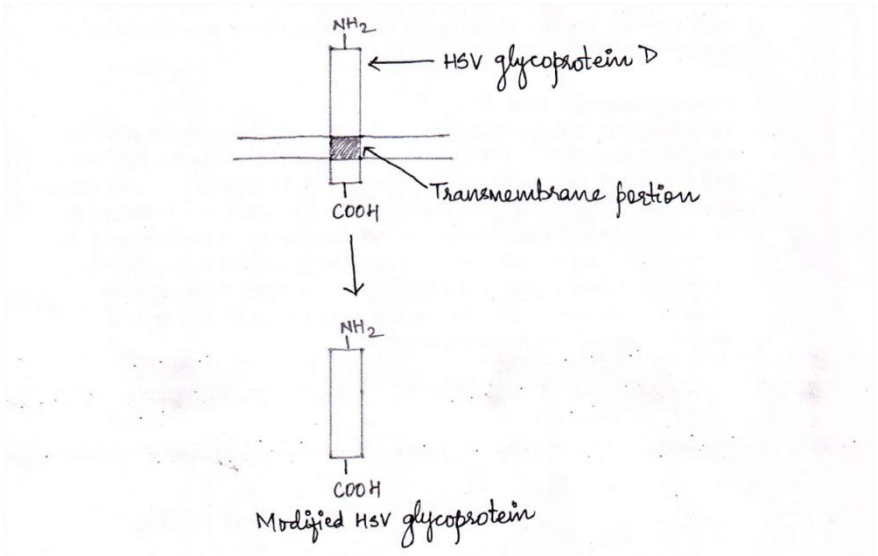
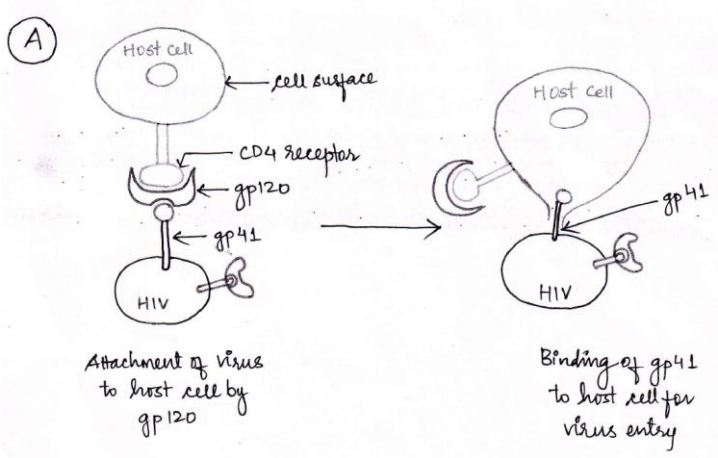


Fig : Modification of HSV glycoprotein D by deleting transmembrane portion (HSV - Herpes simplex virus)

(d) Acquired Immunodeficiency Syndrome (AIDS): The genes for gp120 and gp41 were isolated and inserted into the bacterium *E. coli*. These bacterial cells produce gp120 and gp41 that can be used as recombinant vaccines against AIDS. The gp120 molecules stimulate the host immune system to produce anti-gp120 and antibodies. These antibodies bind to gp120 and prevent its attachment to CD4. In a comparable manner, gp41 molecules also result in the production of anti-gp41 antibodies. These antibodies also bind to gp41 and block the virus host cell union. The net result of using gp120 and gp41 vaccines is that the entry of HIV into the host ceils is prevented.



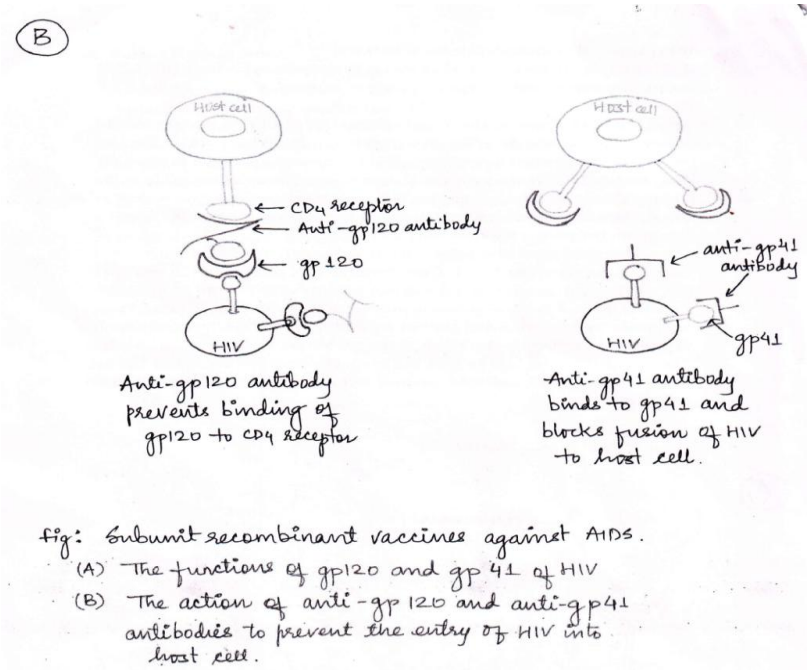


fig: Subunit recombinant vaccines against AIDS.
(A) The functions of gp120 and gp41 of HIV
(B) The action of anti-gp120 and anti-gp41 antibodies to prevent the entry of HIV into host cell.

Answer 8: Molecular Pharming: Molecular pharming is the production of pharmaceutically important and commercially valuable proteins. It harnesses heterologous protein expression systems, such as plants and other traditional expression systems like bacteria, mammalian cell culture and transgenic animals for the large-scale production of recombinant proteins that are therapeutically valuable.

Animal system: The transformation of animals for pharming begins with the preparation of a gene construct in which a specific milk protein promoter is ligated to the gene coding for the protein of interest. Gene transfer is then effected by microinjecting copies of the cloned gene into the proembryo of a recently fertilised egg with the aid of special optics and a micromanipulator system.

Plant system: There are three major transient expression systems to deliver a gene to plant cells: delivery of projectiles coated with ‘naked DNA’ by particle bombardment, infiltration of intact tissue with recombinant agrobacteria (agroinfiltration), or infection with modified viral vectors. Stable transformation is defined by the integration of a target gene into the host plant genome. The generation of transgenic plants uses two principle technologies: *Agrobacterium* mediated gene transfer to dicots, such as tobacco and pea or biolistic delivery of genes to monocots, such as wheat and corn.
Plant expression systems are attractive because they offer significant advantages over the classical expression systems based on bacterial, microbial and animal cells. Firstly, they have a higher eukaryote protein synthesis pathway, very similar to animal cells with only minor differences in protein glycosylation. Secondly, proteins produced in plants accumulate to high levels and plant derived antibodies are functionally equivalent to those produced by hybridomas. Thirdly, concerns about contamination of expressed proteins with human or animal pathogens (HIV, hepatitis viruses) or the co-purification of blood-borne pathogens and oncogenic sequences, are entirely avoided by using plants.

Applications of molecular pharming:

(a) Pharming in mammals:

Milk, blood, seminal plasma and urine are examples of mature system to produce recombinant proteins from transgenic organisms. Milk is the most mature system. ATryn was the first drug approved by FDA in 2009 produced in genetically modified livestock. It is a antithrombin protein purified from the milk of genetically modified goats. Milk proteins are secreted in large quantities by the mammary gland, and this hence becomes the natural bioreactor that enables sustained yield of the target protein. In the laboratory, small animals such as mice and rabbits are used in experiments, but for commercial production, larger animals, usually farm animals such as sheep, goats and cows, are favoured. Many of the target proteins are human proteins such as hormones, enzymes, blood coagulating factors and immunological agents. Protein-based pharmaceuticals already in production, at least in the laboratory, include anti-thrombin, tissue plasminogen activator, anti-trysin, human serum albumin, human alpha-glucosidase, human lactoferrin and various monoclonal antibodies.

(b) Pharming in plants:

Molecular pharming can be used to produce proteins in plants. These proteins can be classified into four broad areas:

(i) Parental therapeutics and pharmaceutical intermediates: This includes all proteins used directly as pharmaceuticals along with those proteins used in the making of pharmaceuticals. Examples are thrombin and collagen (therapeutics), and trypsin and aprotinin (intermediates).

(ii) Industrial proteins: This group includes hydrolases, encompassing both glycosidases and proteases. Enzymes involved in biomass conversion for producing ethanol are candidates for molecular farming.

(iii) Monoclonal antibodies: This group includes all antibody forms (IgA, IgG, IgM, secretory IgA, etc.) and antibody fragments (Fv). They can be produced in plants in both glycosylated and nonglycosylated forms. Transgenic plants have been used for the production of antibodies directed against dental caries, rheumatoid arthritis, cholera, *E. coli* diarrhea, malaria, certain cancers, Norwalk virus, HIV, rhinovirus, influenza, hepatitis B virus, and herpes simplex virus.

(iv) Antigens for edible vaccines: Plant-derived vaccines have been produced against *Vibrio cholerae*, enterotoxigenic *E. coli*, hepatitis B virus, Norwalk virus, rabies virus, human cytomegalovirus, rotavirus and respiratory syncytial virus F. Antigens specific to an individual patient are expressed in tobacco, harvested, purified, and administered to the patient.

Other proteins of medical importance: These include the milk proteins ß-casein, lactoferrin and lysozyme, which would be used to improve child health, and protein polymers that could be used in surgery and tissue replacement. Expression of thioredoxin in foods such as cereal grains would increase the digestibility of proteins and thereby reduce their allergenicity.