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INVESTIGATION ON BIOSYNTHESIS OF SOME BIOSIMILAR DRUGS USING VARIOUS CULTURE TECHNIQUES

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Review Article

Abstract- Biosimilar drugs give a greater prospect in the direction of medical treatment. Biosimilars are highly similar to the already marketed approved drugs in the terms of safety, efficacy and bioavailability. Biosimilar drugs are manufactured with slight modification in manufacturing techniques as compare to synthetic medicine (generic or chemically derived medicine). Biosynthetic potential of plant cell / animal cell utilized to biosynthesized biosimilar drugs. Introduction of new drugs in the form of biosimilars will bring in new drug delivery system. The assurance of high quality, safe and effective medication at reduce cost for the benefit of therapy that in long run are the important key points with biosimilar drugs. India gives 75% of biosimilar market, in which 30 biosimilar products are marketed out of 40 biological products. In 2000, first biosimilar for Hepatitis-B was registered and marketed in India. As of late more than 50 biopharmaceutical items have been confirmed in India, with the greater part of them being biosimilars. The present article focuses on detailed information of biosimilars and plant tissue culture techniques.

Keywords – Biosimilars, Biosynthesis, Plant cells, Animal cells, Plant tissue culture.

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Introduction - A biosimilar (also known as follow-on biologic or subsequent entry biologic) is a biologic medical product that is almost an identical copy of an original product that is manufactured by a different company[1] Biosimilars are officially approved versions of original "innovator" products and can be manufactured when the original product's patent expires.[2]

1.1 Definitions of biosimilars

1.1.1 FDA definition of a biosimilars - The FDA describes biosimilars in the following way: "The biologic product is highly similar to the reference product not withstanding minor differences in clinically inactive components" and that "there are no clinically meaningful differences between the biologic product and the reference product in terms of safety, purity, and potency of the product".[3]

1.1.2 EMA definition of a biosimilar - "A biosimilar is a biological medicinal product that contains a version of the active substance of an already authorised original biological medicinal product (reference medicinal product) in the European Economic Area (EEA). Similarity to the reference medicinal product in terms of quality characteristics, biological activity, safety and efficacy based on a comprehensive comparability exercise needs to be established."
The Marketing Authorisation (MA) application dossier of a biosimilar medicinal product shall provide a full quality dossier together with data demonstrating comparability with the reference medicinal product by using appropriate physico-chemical and in vitro biological tests, non-clinical studies and clinical studies.[4]

1.2 Characteristics of biosimilar

- High molecular complexity
- Quite delicate to changes in manufacturing processes
- Variances in impurities and/or breakdown products can have serious health implications Copies of biologics might achieve differently than the original exclusive version of the Products [5].

1.3 Advantages of biosimilars

- There is huge market needs and growing affordability for Biosimilars in universal and domestic market.
- Development and manufacturing of Biosimilars are improved by existing manufacturing technology [6].
- Due to no investment in phase I-II of clinical trials, Biosimilars are existing at cheaper prices than the reference products, so treatment price with Biosimilars is minor than innovators biological drug [7].

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- The availability of biosimilars may improve access to biopharmaceuticals for more patients and contribute to the financial stability of healthcare systems. Thus, their availability offers potential economic benefit to health care systems while providing global access to the new treatment options for patients brought about by advances in medical science and process engineering [8].

1.4 Disadvantages of biosimilars

- Biosimilars are not as much of stable as chemical-based pharmaceuticals and thus essential cold chain distribution and have a shorter shelf life. This increases the price and complexity of distribution.
- The cost of development will be importantly higher than for chemical based generics.
- The required capital venture in property plant and equipment and the cost of manufacturing will be much greater for Biosimilars than for generic drugs [6].

1.5 Generic v/s Biosimilars

Biosimilars differ from generic drugs by their characteristics, their raw materials and manufacturing processes.

S.no.	Points	Generics	Biosimilars
1.	Description	Chemical origin. Same qualitative and quantitative composition as the active reference.	Biological origin. Same physico-chemical, biological properties as the reference product.
2.	Duration of development	About 3 years.	About 6-9 years.
3.	Development cost	0.5-3 million dollars.	40-80 million dollars.
4.	Manufacturing process	Manufacturing processes of generic is the same as bioavailability.	Biosimilar method of manufacturing differs from that of the reference product at several levels.
5.	Marketing authorization dossier	Abbreviated Dossier. Data from bioequivalence and bioavailability.	Complete application (Phase I and III (IV)). Comparative preclinical and clinical data in terms of quality, efficacy and safety versus the reference product.

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6.	Therapeutic indication	All the indications of the originator are also indications of generic.	Record indication by indication, and the need for setting up clinical trials for each of them if they are multiple, but exceptions exist.
7.	Substitution	Authorized.	Not authorized.
8.	Reimbursement	Reimbursement rate identical to that of the originator.	Reimbursement rate identical to that of the originator.
9.	Commercial promotion	Easier to implement and less expensive than the reference drug.	Long and costly, is close to that originator.

Table - I: Essential differences between the two regulatory pathways relating to biosimilars and generics.[9]

1.6 Nomenclature of biosimilars

The World Health Organization (WHO) and the FDA have been working for years on the non-proprietary naming of biosimilars. In August 2015, the FDA published a draft guideline on the topic.[10] In brief, the guideline calls for the assignment of a four character alphabetic suffix to the non-proprietary name of the original product to distinguish between innovator drugs and their biosimilars.[11] The WHO INN system calls this suffix a biologic modifier.[12]

1.7 Biosimilar regulatory pathways and guidelines

In 2004, the EU was the first region in the world to establish a legal framework and regulatory pathway for biosimilars, with the first biosimilar medicine approved by the European Medicinal Authority (EMA) in 2006.[13,14] The World Health Organization also issued biosimilar guidance,[15] which shared scientific principles with the EMA guidance and served as a ‘‘roadmap’’ for guidance documents being developed by other countries, including Australia, Canada, Japan, Turkey, Singapore, South Africa, and Taiwan. In 2009, the US Biologics Price Competition and Innovation Act was passed, and 3 biosimilar guidance’s were released in 2012. [16] In the EU, a biosimilar is a copy version of an already authorized biotherapeutic with demonstrated similarity in physiochemical characteristics, efficacy, and safety, based on a comprehensive comparability exercise. [13,14]

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1.8 The regulation of biosimilars is an evolving process

a. European Medicines Agency (established the first regulatory framework for assessing biosimilars)

b. Food and Drug Administration **(** In March 2010, the US Congress passed legislation creating a legal pathway for biosimilars under the Patient Protection and Affordable Care Act, the legislation providing an approval pathway for biosimilar biological products is outlined in section 'Title VII – Improving Access to Innovative Medical Therapies: Subtitle A – Biologics Price Competition and Innovation'.[17])

c. World Health Organization **(**In April 2010, the WHO Expert Committee on Biological Standardization published final 'Guidelines on Evaluation of SBPs' (similar biotherapeutic products), as part of its mandate to assure global quality, safety and efficacy of biotherapeutics. [18])

Other regulatory agencies in the world

a. Australian Therapeutic Goods Administration (In June 2006, adopted the European guidelines for registration and approval of biosimilars.[19])

b. Canada (In March 2010, published revised submission requirements for 'subsequent entry biologics' (SEB) that largely follow EMA guidelines. [20])

c. The Japanese Ministry of Health, Labours and Welfare (In March 2009, issued guidelines for the approval of biosimilars.[21] In October 2009, Japan approved a somatropin human growth factor biosimilar. [22]

S.No.	Regulatory bodies	Terminology used for Biosimilar
1.	US-FDA	Follow on Biologics
2.	WHO	Similar Bio therapeutic Product
3.	India	Similar Biologics
4.	Europe	Biosimilar
5.	Brazil	Follow on Biologics
6.	Canada	Subsequent Entry Biologics
7.	Japan	Follow on Biologics

Table-II: Various biosimilar terminologies used by different regulatory bodies. [23]

1.9 Regulatory framework in India

The regulatory bodies responsible for approval of 'similar biologics' in India are the Department of Biotechnology (DBT – under the Ministry of Science and Technology).[23]

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The competent authorities associated with in the approval process are as follows:

Review Committee on Genetic Manipulation (RCGM)

Genetic Engineering Appraisal Committee (GEAC)

Central Drugs Standard Control Organization (CDSCO)

1.9.1 Biosimilar approval pathway in India

The general regulatory pathway for the approval of the biosimilar in India is shown in figure. The regulatory pathway of biosimilar is bit different from the normal pathway for their approval. [24]

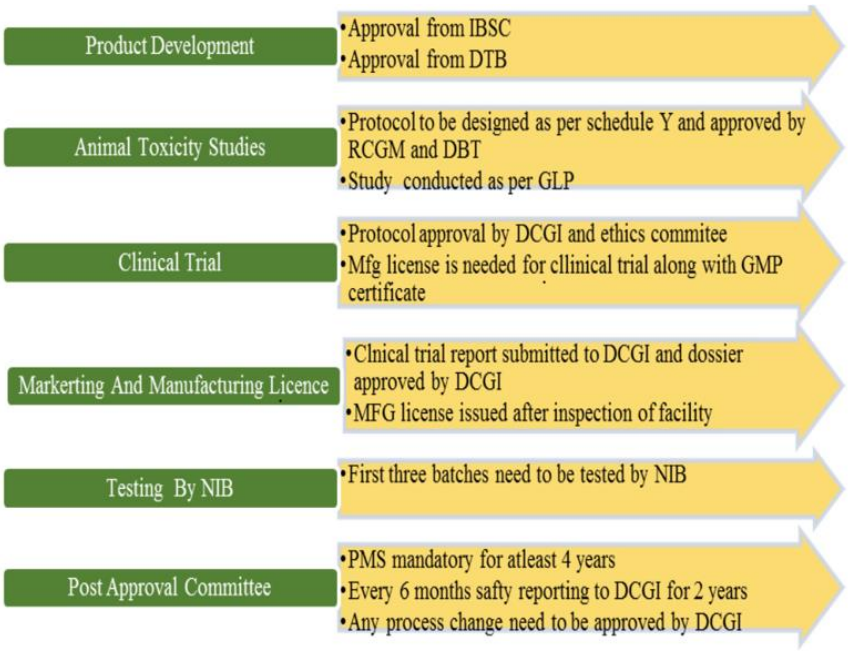

Fig-I: Approval pathway of biosimilars in India

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1.9.2 Top biosimilar products in India

S.No.	Product Name	Active Drug	Manufacturer	Indication/ Disease
1.	Intacept	Etanercept	Intas	Rheumatoid Arthritis, Psoriatic Arthritis
2.	Epofer	Epoetin Alfa	Emcure	Anaemia
3.	Grpfeel	Filgrastim	Dr. Reddy's	Neutropenia
4.	Rituxirel	Rituximab	Reliance Life Science	Non-Hodgkin's Lymphoma, Rheumatoid Arthritis
5.	Exemptia	Adalimumab	Zydus Cadila	Ankylosing Spondylitis

Table-III: Top biosimilar products in India

1.10 Clinical trial development for biosimilars

To meet the FDA requirements, clinical development of the biosimilar begins with studies to demonstrate comparable pharmacokinetics (PK) and pharmacodynamics (PD) with the reference product in a relevant population.[25] Also included in early clinical development are investigations that focus on safety, including immunogenicity. Once PK, PD, and immunogenicity similarity to the reference product has been demonstrated, at least one phase 3 clinical comparability trial is conducted to confirm similar efficacy and safety in a sensitive population.[26]

S.No.	Key Considerations in Evaluating Phase 3 Clinical Studies of Biosimilars	
1.	Comparability	An equivalence design at the 90% or 95% confidence interval is used (generally preferred to a noninferiority design). An equivalence design establishes that the biosimilar is neither superior nor inferior to the reference product.[27]
2.	Patient population	Should be clinically relevant. Does the study use the most sensitive patient population, that is, the population in which clinically meaningful differences in safety and effectiveness between the biosimilar and reference product are most likely to be detected.[27]

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3.	Power/sample size	Study is sufficiently powered to detect potential differences between biosimilar and reference product. [27,28]
4.	Dose	The dose and route are consistent with the reference product.[27]
5.	End points	End points are relevant to the disease state and sensitive enough to detect clinically relevant differences in efficacy and safety, if any, between the biosimilar and reference product. [27]
6.	Study duration	The duration of the study was appropriate to detect clinical effects. [27,28]
7.	Statistical analysis	A per-protocol analysis includes only patients who followed the protocol, whereas an intention-to-treat analysis includes all randomized patients. If the study used an equivalence design, a per-protocol analysis was used. [29,30]
8.	Efficacy	Are efficacy measures within the prespecified acceptable margin of equivalence? [27]
9.	Safety	Are the incidence and types of AEs comparable between biosimilar and reference product? [27]

Table-IV: Key considerations in evaluating phase 3 clinical studies of biosimilars.

1.11 Application of biosimilars

Biosimilars in oncology practice - Focus on biosimilars in oncology practice, where they are not used simply for the replacement of hormones (e.g. growth hormones, insulin) or the treatment of renal insufficiency (i.e. erythropoietin); but as supportive therapy for immunosuppressed patients receiving multiple cycles of cytotoxic therapy, or for healthy stem cell donors who obtain no direct therapeutic benefit from treatment. In general, oncologists should be aware that the terms 'biosimilar', 'similar biotherapeutic product', 'subsequent entry biologic' or 'follow-on biologic product' refer to the same type of product. Furthermore, it is important to have a detailed knowledge of the characteristics of these products, including extrapolation, substitution, labelling, traceability, safety and immunogenicity.[31]

Biosimilars in rheumatology - The first biosimilar monoclonal antibody to infliximab (CT-P13) is concerned, which was approved for marketing in South Korea for all the six indications of infliximab, the approval was based on a single equivalence trial conducted in patients with RA, and supplemented by a pharmacokinetic study in patients

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with AS. They showed that infliximab and its biosimilar result are not different in terms of clinical efficacy, safety and immunogenicity, both 188 at 30 and 104 weeks. The Italian Society of Rheumatology states that biosimilars should be limited to the indications for whom the "comparability test" was executed. Any claim must be validated with specific clinical trial, in particular for the extension of use of biosimilars in axial spondylarthritis, enteropathic or psoriatic arthritis, and, overall, paediatric patients. Validation should be conducted by direct comparison of the results coming from well-designed clinical trials on the innovative product and the original treatment. This would result in a great potential for the appropriate use of biological therapies in paediatric rheumatic diseases and enteropathic arthritis, in terms of management of the disease, and in terms of cost reduction

Biosimilars in dermatology - The introduction of biological therapeutics for the treatment of chronic plaque Pso has significantly improved several important patient outcomes including quality of life, and allowing an effective long-term control of the skin disease. In particular, cyclosporine induces nephrotoxicity and hypertension, methotrexate liver damage, and acitretin can result in skin and mucosal toxicity. Currently approved biologics for the treatment of chronic plaque Pso include the TNF-alpha inhibitors adalimumab, etanercept and infliximab, and ustekinumab, an anti-IL-12/23 monoclonal antibody.

Biosimilars in inflammatory bowel disease - Infliximab and adalimumab are the only biologics currently approved for the treatment of Crohn's disease (CD), and, together with golimumab, for ulcerative colitis (UC) in the European Union (EU). In the Western countries outside EU, also certolizumab pegol and natalizumab are approved for Crohn's disease. At present, two infliximab biosimilars have been filed for the EMA, Inflectra®, and Remsima®, although there are currently no studies comparing infliximab and biosimilars in IBD.[32]

1.12 Worldwide scenario of biosimilars

Presently, there is just a predetermined number of biosimilar in the market, confined to the classes of development hormones, monoclonal antibodies, combination proteins, interferons, and low-atomic weight heparins. The 60+ biosimilar in the development pipeline include medications in therapeutic areas such as oncology, immunology, and diabetes, with biosimilar producers showing particular interest in leading Biologics with recent or pending patent expiry. As biosimilars are broadly utilized in treatment of a diabetes, tumours, CVDs, immune system illnesses, rheumatoid joint pain, kidney disappointment. India shares 75% of biosimilar market, in which 30 biosimilar products are marketed out of 40 biological products. First biosimilar was registered and marketed in India for a hepatitis B in 2000. As of late more than 50 biopharmaceutical items have been affirmed in India, with the greater part of them being biosimilar.[33]

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1.13 Plant tissue culture

Plant tissue culture is a technique used for *in vitro* regeneration of plants. It relies on maintaining plant cells in aseptic conditions on a suitable nutrient medium. The culture can be sustained as a mass of undifferentiated cells for an extended period of time or regenerated into whole plants. Plant tissue culture techniques are also central to innovative areas of applied plant science, including plant biotechnology and agriculture. Callus culture and suspension culture are the basic technique used to produce the desired metabolites of plants. The plant and tissue cultures have been enabled to increase the knowledge in many areas including differentiation, cell division, cell nutrition and cell preservation but now, cells are cultivated *in vitro* in bulk or as clone from single cells to grow whole plants from isolated meristem, then induce callus and develop complete plantlets by organogenesis or by embryogenesis.[34]

1.13.1 Plant tissue culture - Important terms

The important terms in tissue culture systems are given below:

1.13.1 .1 Callus culture - The growth and maintenance of unorganised cell masses initiated from disorganised growth of pieces of plant tissues or the explant are called callus. Callus cultures are initiated from a small part of an organ or tissue segments called explant on a growth supporting solidified nutrient medium under sterile conditions. Any part of the plant organ or tissue may be used as the explant. Typical explants are leaf, root, stem-nodal and internodal parts, axillary buds, shoot tip, shoot apical meristem, seeds etc.

1.13.1 .2 Suspension culture - Suspension culture is the culturing of isolated cells in a liquid media. The callus is removed from the original explant and transferred to a medium and placing it on a mechanical orbital platform shaker at 100-150 rpm. Agitation is required for suspension cultures for three purposes: it serves to break up the cell aggregates; it maintains a uniform distribution of the cell of various sizes and shapes, and cell clump in medium ; and it provides gas exchange for the cell to sustain cell respiration in the liquid medium.[35]

1.13.1 .3 Tissue culture medium - Growth and development of explants in vitro are products of its genetics surrounding environment and component of the tissue culture medium. Tissue culture medium consist of 95% water, macro and micronutrients, plant growth regulators, vitamins, sugars and sometime various other simple to Complex organic materials.[36]

1.13.1.4 Aseptic conditions - All glassware's, culture vessels, instruments, nutrient media used during protocol of tissue culture must be aseptic. The nutrient media is sterilized in autoclaving. The sterilization of glassware's and

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metallic instrument can be carried out in dry heat as well as moist heat i.e., either in hot air oven or autoclave. The sterilized explants are then aseptically transferred to culture vessels.

1.13.1 .5 Surface sterilization - Surface sterilization of explant is explant is most important in tissue culture. The commonly used surface sterilizing agents are sodium hypochlorite (1-2%), bromine water (1-2%), hydrogen peroxide (10-12%), mercuric chloride (0.1-1%) and silver nitrate (1%).

1.13.1 .6 Measurement of growth - The growth of cells in suspension can be followed by the increase in dry weight or increase in total volume, although cells can be counted and growth thus determined using a haemocytometer.

a. Cell counting - A cell suspension in a balanced salt solution is taken and using a Pasteur pipette, a small amount of cell suspension is transferred to both chambers of a haemocytometer and a coverslip is used to allow each chamber to be filled with capillary action. Starting with one chamber of the haemocytometer, all the cells in the 1-mm centre square and four 1-mm corner squares are counted (Fig-II) in each chamber.

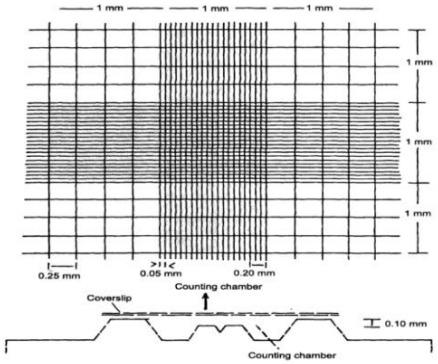

Fig-II : Diagrammatic view of a haemocytometer under the microscope.

.Each 1-mm square of the haemocytometer, with coverslip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to 1 ml, the cell concentration per ml (and the total number of cells) will be determined as follows:

Cells per ml = the average count per square × dilution factor × 10^4

Total cells = cells per ml × original volume of culture medium from which cell suspension was taken.

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***b. Packed cell volume* [PCV]** - PCV is correlated with fresh weight growth. To determine PCV, a known volume of uniformly dispersed suspension is transferred to a 10/15 ml graduated centrifuge tube and centrifuged. Then PCV is expressed as ml pellet per ml culture.

c. Cell fresh weight - Cells are collected on a pre-weighed (in wet condition) circular filter paper in a Buchner funnel. The cells are then washed with water to remove the medium under Vacuum, and weighed. Growth is expressed in fresh weight per ml medium.

d. ***Cell dry weight*** **-** Cells are collected as above using a pre-weighed filter paper and they are dried for 12 hours at 60º C and cooled in a desiccator (to avoid any increase in weight due to moisture during cooling) and re-weighed. Growth is expressed as dry weight per ml medium.

e. Non-invasive method - This does not require withdrawal of culture samples from the flask. Bloom (1992) described a method where a culture flask fitted with a ruler is kept for 5 minutes at an angle of 30º-60º. A change in the height of the sediment at different time periods is an indication of growth.[35]

1.13.1 .7 Growth index (GI) -

Growth index is calculated by the ratio of the total mass transferred and final volume accumulated during propagation of the culture. In more actual terms growth index is the measurement of the final and the initial masses at sampling time, which is represented as follows:

$$GI = F_m - I_m / F_m$$

where GI is growth index, and *F*m and *I*m represent the final and initial masses, respectively (either as fresh or dry weight).

1.13.1 .8 Growth Curve **-** Cells in suspension can exhibit much higher rates of cell division than those in callus culture. Suspension cultures when maintained under controlled conditions of light, temperature, and aeration follow a predictable pattern of growth curve (Fig-III)

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***a. Lag phase*:** The culture first passes through a lag phase in which there is little growth. The lag phase is the period when the cells adjust to the replenished supplies of nutrients and undertake all the necessary synthesis prior to cell division.

b. Log phase: The cultures then pass through the logarithm phase or exponential phase of growth in which cells divide very rapidly, causing a logarithmic increase in cell number. Under optimum conditions, cell numbers double every 20–50 h, depending upon their species. The culture passes through a further period of rapid cell division that results in a linear increase in number, slowing at the phase as some nutrients become limiting.

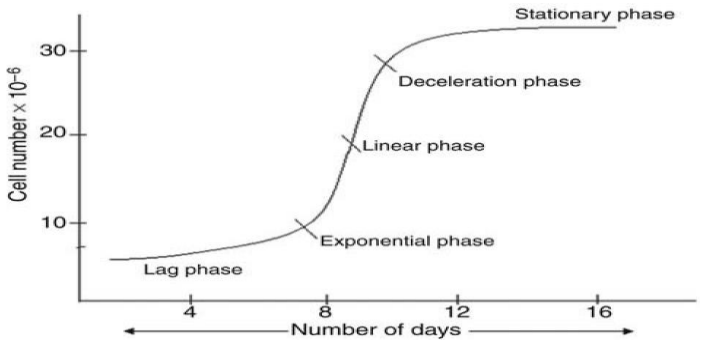

Fig-III: Growth curve showing different phases of growth

c. Stationary phase: The cultures then reach a stationary phase, when the rate of cell division within the culture decreases, and the cell number is stabilized and growth finally halts. As nutrients are depleted, some of the cells of the culture begin to show senescent characteristics and a low level of cell division will maintain cell numbers. If the cells are left in the stationary phase too long, they will die.[37]

***1.13.1.9 Viability measurement* -** Determining viability of cells in suspension cultures by following method.

***a. Fluorescein diacetate* (FDA) -** FDA is added to a few drops of cell culture and the cells are observed under a fluorescent microscope. FDA itself does not fluoresce. Once inside the cell, it is cleaved by esterase activity and green-glowing fluorescein is released. Fluorescein is not freely permeable across the plasma membrane and it accumulates in living (but not dead) cells.

***b. Evans blue Stain* -** Evans blue stain is excluded by living, functional membranes. Thus, it is taken up by dead cells and excluded by living cells. This is easily seen under the light microscope [35].

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1.14 Applications of tissue culture for biosimilars

The use of in vitro tissue culture remains a feasible strategy for the production of structurally complex and high-value natural products. The production of pharmaceuticals using plant culture systems can offer significant advantages, including reduction in costs, rapid production, low burden of human pathogens and scalability. In the early 1990s, transgenic plants were endorsed as an alternative means of production of pharmaceutically important proteins. A transgenic system offers several advantages, including decreased costs, increased ease of delivery and scale-up, decreased risk of contamination with animal and human pathogens, and eukaryotic protein processing. Table-8 list's some pharmaceuticals produced in plants.

S.no.	Plant-made pharmaceutical	Plant,	Use
1.	ELELYSO™ (taliglucerase alfa)	Carrot or tobacco cell culture	Enzyme replacement
2.	Vaccine (NDV)	Tobacco suspension cultures	Against Newcastle disease virus
3.	VEN150	Rice seeds	For HIV-associated chronic Inflammation
4.	Moss-GAA	Moss	Pompe disease
5.	CaroRx	Tobacco leaves	For dental caries
6.	PBI-220	Tobacco leaves	Antibody for anthrax

Table-V: List of some pharmaceuticals produced in plants. [38]

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