

Plant cells and suitable bioreactors

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Abstract:

This study is based on knowledge that I acquired at Advanced training course 2015 about cell expansion and protein expression in standard and single-use bioreactors at Zürcher Hochschule für Angewandte Wissenschaften Zurich, further elaboration and discussion of this knowledge.

It consists of three distinct parts, the first part explains the basic knowledge necessary for expansion of plant cultures in bioreactors, discussing characteristics of plant cells, growing conditions, environments, culture type and culture type mode.

A second part based on the explanation of main characteristics of bioreactors that are suitable in the industry and market development as well as their advantages and disadvantages.

Finally, the last part is a bibliographic research about bioreactors in order to achieve which is the most suitable device to grow plant cells Bright-yellow 2 of *Nicotiana tabacum*, cells, which are used for the production of recombinant proteins.

Resum

Aquest estudi, es basa en l'aprenentatge i l'ampliació dels coneixements que vaig adquirir a l'Advanced training course 2015 sobre el creixement de cèl·lules i expressió de proteïnes en reactors d'un sol ús, al Zürcher Hochschule für Angewandte Wissenschaften de Zürich

Consta de tres parts diferenciades, la primera part explica el coneixement bàsic necessari per poder fer créixer cultius vegetals en bioreactors, es parla de característiques de les cèl·lules vegetals, condicions de cultiu, medis, tipus de cultiu i tipus de mode de cultiu.

La segona part es basa en l'explicació de les característiques dels bioreactors que hi ha al mercat per indústria i desenvolupament, així com els seus avantatges i desavantatges.

Finalment, l'última part és una recerca bibliogràfica per saber quin de tots els bioreactors és el més indicat per fer créixer cèl·lules vegetals Bright-yellow 2 de la *Nicotiana tabacum*, cèl·lules que s'usen per l'obtenció de proteïnes recombinants.

Discussion of areas

As its commented in the abstract, this essay is based on knowledge that I acquired in Switzerland but, it was possible to understand and continue their classes thanks to a different subjects which I had done in the University. I based the concordance of this study to these subjects.

It seems clearly that this essay is focused to the area of vegetal physiology due to their constant uses of their terminology and knowledge, such as information of cultures, bioreactors, plant cells, and plant cells characteristics. Not only I needed in Switzerland, also I have been to bury me in this subject.

Other necessary area for this molecular biology, it's indispensable to know about cells transformation, genetics, and biotechnology.

Last but not least, botanic are necessary, botanic are defined as classify, knowledge of anatomic structures and functions of vegetal, consequently, is basic for the third and last part of this essay.

Objectives

Explain the basic knowledge necessary for expansion of plant cultures in bioreactors

Analyze of main characteristics of bioreactors that are suitable in the industry and market development as well as their advantages and disadvantages.

Bibliographic research about bioreactors in order to achieve which is the most suitable device to grow plant cells Bright-yellow2 of *Nicotiana tabacum*.

Hypothesis

The first and second part of this study, are only bibliographic, so as a result, a hypothesis is not needed, but in third part, where a question has to be answered (which bioreactor is the most suitable device to grow plant cells Bright-yellow2 of *Nicotiana tabacum*) it's necessary a hypothesis. Based in my learning in Switzerland, it seems that bioreactor wave type, is the most suitable.

1. Basic knowledge

1.1 Introduction to plant cells

The use of plant cell and tissue cultures for production of biologically active substances is called plant cell-based bioprocessing; the active substances that this technique allows was low molecular secondary metabolites and recombinant proteins.

Plant cell-based bioprocessing has some significant advantages over the traditionally grown of the whole wild plant or transgenic plant. The most important advantage is the sterile production of metabolites under defined controlled conditions, this means that climatic changes and soil conditions are not able to influence the product yield and quality.

Plant cells have plenty of advantages over mammalian cells, insect cells, and bacteria; these cells are capable of performing complex posttranscriptional processing, which is a precondition for heterologous protein expression. When they are compared with mammalian cells (which dominate the commercial protein manufacture) plant cell cultures have lower cost and are safer due to the lower risk of contamination by viruses, pathogens and toxins. Also plant cells grow at room temperature (25-27°C), need a lower aeration (0.1-0.3 vvm) than microbial culture and they have a lower shear stress than mammalian cells due the cell wall.

Characteristic	Microbial culture	Plant cell culture	Mammalian cell culture
Size	2–10 μm	10–100 μm	10–30 μm
Individual cells	Often	Often aggregates	Sometimes adherent
Inoculation density	Low	High (10%)	High (5–10%)
Growth rates	Rapid ($t_d = 1-2$ h)	Slow ($t_d = 2-7$ d)	Slow ($t_d = 20-50$ h)
Shear sensitivity	Low	Moderate	High
Stability	Stable	Unstable	Unstable
Product accumulation	Intra-/extracellular	Mostly intracellular	Mostly extracellular
Culture medium	Often simple	Often complex	Complex
Temperature	26–36 °C	25–27 °C	29–37 °C
Aeration	Often high (1–2 vvm)	Low (0.1–0.3 vvm)	Low (~ 0.1 vvm)
Foaming	Often high	sometimes foaming	Sometimes foaming
pH-value	3–8	5–6	7.0–7.4
Cell density	(Very) high	Low	Low–middle
Scale-up	Easy	Difficult	Difficult

Figure 1: Differences between cultures

Source : [1]

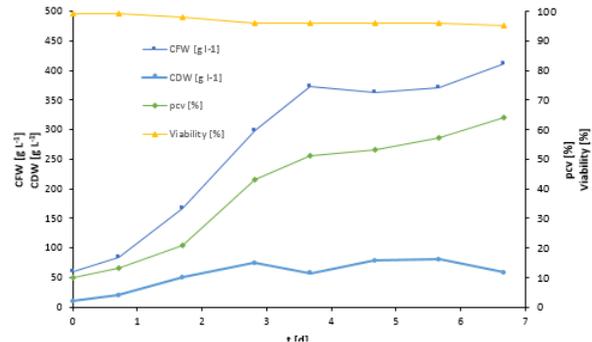
Compelling disadvantages which scientists have to deal, are the difficult scale-up, the low cell density, the intracellular product accumulation (mostly in vacuoles), but the most significant disadvantage is the slow growth rate with a t_d (division time) of 2 to 7 days.

1.2 Plants cells characteristics

Eukaryotic systems, like plant cells, have the ability to produce secondary metabolites and glycoproteins, which the pharmaceutical industry uses for creates recombinant proteins and secondary metabolites which are used in both industries pharmaceutical and cosmetic.

Sigmoid curve ideally represent the cell growth, with lag, exponential, delay, stationary growth and lethal phases. [Graphic 1]

In addition, plant cells have totipotency (potential to form all cell types and a whole plant), as is said before, plant cells have a doubling times of days to weeks due to their large size (100 μm). In exponential growth, recombinant proteins are occasionally secreted into the culture medium, and secondary metabolites are accumulated in the vacuole or released to the medium in the stationary growth.



Graphic 1: BY-2 sigmoid growth

Source : Own.

The robustness of plant cells is moderate and can be attributed to the cell wall, this robustness have some variations in order to age of the culture, species and culture type, they have lower shear stress, and can be cultivated in reasonable agitation and aeration conditions.

1.3 Culture conditions

Mostly of the plant culture cells have similar culture conditions as temperature, between 25 and 27°C, a medium pH with an optimal parameter of 5.0 and 6.0. Also culture cells need some aeration; this aeration is extremely lower than microbial systems and comparable to mammalian cultures [Figure 1]. Some cultures have periodic dark/light cycle of 8 h and 16 h or continuous introduction of light (0,6 -10 klux or 80,7-1345 $\mu\text{mole m}^{-2} \text{s}^{-1}$), mammalian cells have to be cultivated in dark as the consequence of the light-sensitive compounds of their media culture.

In order to eliminate long lag phases (120 hours) plant cell cultures can be initiated with high cell concentrations (10% of culture volume).

1.4 Media

Nutrient supply is a critical element when culturing plant cells. The main substance is doubled distilled and deionized water which represents 95% of the media. All culture media have a basal medium with carbon source like fructose, sucrose, glucose and sorbitol, also with organic supplement and inorganic supplements; in organic

supplements as amino acids, vitamins and cofactors as tocopherol are used. The inorganic supplements are macro and microelements, the microelements are in μM concentrations and macroelements in mM. [Figure 2]

In addition nutrient supply needs some phytohormones as growth regulators like auxins, cytokinins and gibberellins. Growth regulators affect the growth process, cytokinins such as adenine which promotes cell division and auxins, like indole-3-acetic acid (IAA), or 2,4-dichlorophenoxyacetic acid (2,4-D) which is used as a dedifferentiating hormone for rapid callus induction. With low auxin concentration and a high cytokinin concentration the cell growth are stimulated and switched concentrations cell division are promoted.

Water	Basal medium			Phytohormones (growth regulators)	Support matrix
	Carbon source	Organic supplements	Inorganic supplements		
	Sucrose	Amino acids: Ala, Arg, Asn, Cys, His, Ile, Leu, Met, etc.	Microelements in μM concentrations: Fe, Mn, Zn, Cu, Mo, I, B, Co	Auxins: IAA, 2,4-D, NAA, IBA, 2,4,5-T	Agar
	Glucose	Vitamins and cofactors: myo-Inositol, thiamine, pyridoxine, folic acid, ascorbic acid, tocopherol, yeast extracts ^a	Macroelements in mM concentrations: N, S, P, K, Mg, Ca	Cytokinins: kine- tin, BAP, zeatin, purine, adenine	Agarose
	Fructose			Coconut milk or coconut water ^a	Gellan gums
	Sorbitol			Gibberellines ^a : GA ₁ , GA ₃ , GA ₄ , ABA ^a	

^aOccasionally used

Figure 2: Medium composition

Source : [1].

Recombinant protein production is influenced by supplements which stabilizes the proteins like bovine serum albumin, PVP (polyvinylpyrrolidone), gelatin and sodium chloride. These components are used in medium MS developed by Murashige and Skoog for tobacco cultures, among others. [2]

Finally if a solid support matrix is needed, the addition of agar, agarose, and gellan gums can reach this purpose. In the below table the constituents of plant cell culture media are summarized.

1.5 Cultures

Industry and research have different culture types such as callus, plants cell suspension, hairy root cultures, embryogenic and shoot cultures. *Nicotiana tabacum* BY-2 growth in plant cell suspension, due to this reason this report only explains in profundity cell suspension, although other culture types are explained too, but only as a little review.

1.5.1 Callus cultures

Callus cultures are fundamental in vitro culture of plants cells, not only for acquire a high density of biomass but also because they are necessary for the establishment of cell suspensions.

Callus is described by an amorphous mass of unorganized parenchyma cells, the formation of callus normally is achieved by placing the explant on an appropriate solid growth medium with the necessary components such as phytohormones in a Petri dish incubated at 25°C in darkness or low light.

Callus cultures have a developmental stage of cells described as dedifferentiated cells (DDC, restored totipotency). A DDC callus has friability, a soft cell mass with fast exponential growth. If a hard callus is achieved, Evan's method can reestablish a friable callus with addition of phytohormones like auxins, pectinase or shorter subculturing intervals.

Callus has particular maintenance and culture conditions, with a doubling time around 7 days; the subcultivation is necessary every 3 to 4 weeks (depending on species).

1.5.2 Hairy roots

Hairy roots (transformed roots) are generated by the transformation of plants or explants with agropine and mannopine-type strains of *Agrobacterium rhizogenes* (a gram negative bacterium). Neoplastic roots are the developmental stage of cells and roots covered with tiny hairs are the appearance, a medium liquid or solid can achieve high densities of this culture.

With a doubling time between one to eight days a subcultivation every two to four weeks is necessary.

Hairy roots are genetically and biochemically very stable with a high integrity, but this type of culture has a shear-sensitive tissue. Consequently, it is difficult to have cultures in vitro cultivation (is required a special bioreactor design) and are also complicated the scale-up procedures.

1.5.3 Embryogenic and shoot cultures

Embryogenic and shoot cultures are used for micro propagation and plant breeding, and like hairy roots, they belong to the group of differentiated organ cultures. These kind of cultures are established via meristem, seed germination or embryonic culture.

1.5.4 Plants cell suspension

As commented above, *Nicotiana tabacum* BY-2 cells grow in plant cell suspension and also, the growth of a callus is necessary for this type of culture. The callus produced is dispersed by inoculating the fragments of callus in a liquid medium, and if it's necessary to achieve friability in the callus, the Evan's method is required. After callus isolation and subcultivation the culture is usually filtered with a sieve of 0,3mm to 0,5 mm in order to remove large aggregates.

Homogenization aims to produce a well-mixed suspension. Established plant cell suspension is generally maintained in shake flasks at 25°C and between 110 to 120 rpm. With a doubling time of 0.6 and 5 days plant cell suspensions grows faster than callus culture.

Plant cell suspensions are highly heterogeneous as variability in terms of morphology (cell size, cell shape, and cell aggregation), rheological characteristics, growth and metabolic pattern.

Rheological characteristics are important in the process of scale-up; plant cell suspensions are non-newtonian fluids (with plastics and pseudoplastics characteristics), so the density of this cultures raises proportional to cell density. As a result plant suspension cells very rarely grow as single cells, they form aggregates based on cell adhesion and results from the secretion of wall extracellular polysaccharides, which prevent cell separation.

1.6 Transformed plant cell cultures

One of the cell lines of *Nicotiana tabacum* BY-2, expresses the tumor-specific human antibody M12. This cell line was generated by Agrobacterium-mediated stable transformation. [3]

First of all, neither plant cells cultures nor cells nor plants are transformed, they are wild type. Now non-transformed plant cell cultures are used only if the natural biosynthesis pathway of the target compound has to be examined or if the target compound is naturally synthesized or expressed in a reasonably high amount (such as rosmarinic acid).

Transformed cell cultures, exist in order to optimize the production of a target compound with a stable transformation (all the generations of this plants have the

same expression) or with a transient transformation (a few generations have the transformed form).

1.7 Examples of different culture modes

Not only bioreactor type is important to achieve a high density of product, also it is necessary to determine the culture mode; batch, fed-batch and continuous cultures such as chemostat perfusion and dialysism are some examples of different culture modes. [4]

1.7.1 Batch

Batch processes constitutes the classical bioreactor mode, infrequently applied in cell culture processes but used in microbial bioreactors such as fermenters. A closed system with a constant culture volume is harvested at maximum cell density or product titre.

A few advantages of this mode is the simplicity and flexibility; these characteristics lead to an easy scale-up, without contamination, are cheaper and have shorter or less steps. But low space-time yield and problems in process characterized by inhibition of substrate and product (less production) are some of the most important disadvantages of this mode and make him useless.

1.7.2 Fed-batch:

This kind of batch is often used in industrial applications; with this system, industries can reach higher living cells densities and product titres compared to batch cultivations. Exist three different versions: One with a culture volume increased continuously (feeding), other with a culture volume kept constant so using and exchange and feeding, and finally a repeated fed batch this one after fed batch-phase, cell suspension is partially harvested and fresh medium is fed to the system. The main advantage of this model is the higher cell concentration that can reach and the no limitation of high production, but is complex, expensive and the system has to be developed.

1.7.3 Continuous

Is an opened system with a constant culture volume and the process runs in a batch mode up to desired cell density which differs between different species. The subsequent introduction of continuous mode is based on a continuous medium feed and withdrawal of culture broth. Two different processes can be used in continuous batch, without cell retention and with cell retention, with cell retention is called perfusion.

Different advantages such as high space-time yields (months) and smaller bioreactors, lower investment costs for processes with cell retention; never cell inhibition by product (extracellular) or substrate happens and present high sterile environment. In the other hand needs a higher levels of instrumentation, higher level of consumption of culture medium and costs of down-streaming, also exist a probable wash out of cells (elutriation) or nutrient limitations when dilatation rate is too high or too low and finally more complicated validation and registration procedures. Also it is laborious, and has a high duration.

2. Bioreactors:

A bioreactor is defined as a closed system (vessel/bag or apparatus) in which a biochemical reaction involving biocatalyst takes place [1]. In the process the biocatalysis is converted into an expressed protein which is biomass or expressed proteins, it should be pointed out that the term “fermenter” is used only by bioreactors which involves fast growing microorganisms, but in American English this term is used in both bioreactors.

The primary role of a bioreactor is to provide containment with sustainable conditions for cell growth and/or product formation. In general a cell culture bioreactor has to meet the following demands:

Guaranteed cell-to-cell contact and a surface for cell detachment in case of anchorage-dependent growing cells.

- Homogeneous and low-shear mixing and aerations.
- Sufficient turbulence of effectual heat transfer.
- Adequate dispersion of air and gas.
- Avoidance of substrate segregation.
- Measurability of process variables and key parameters.
- Scale-up capability.
- Long-term stability and sterility.
- Easy of handling.
- Reasonable maintenance.
- Avoids all possible contamination of the culture.

Before describing all the bioreactors it's imperative to know a few characteristics about how to determinate grow of the plant cells and critical culture points in this kind of devices.

2.1 In-Process control (IPC), determination of plant cell growth:

Process monitoring and control are generally facilitated by standards bioreactor facility which uses pressure, temperature, pH gas flow rate, pO₂ (dissolved oxygen), pCO₂ (dissolved carbon dioxide), and conductivity sensors.

Not only in bioreactors IPC is required, also in maintenance of plant cell cultures due to the high quality cell culture material which is constantly available, this maintenance is carried out with high viable cells (<90%) at regular time intervals. A detailed knowledge of the plant cell culture is required such as inoculum density or if cells are genetically modified or not.

To characterize and design bioprocesses based on plant cells with secondary metabolite production or protein accumulation/ secretion and nutrient utilization, cell growth must be determined

Plant cell growth must be determined in both processes such as experimental amounts or in scale-up productions, normally is measured as fresh weight or as dry weight. Fresh weight values (expressed in g) are obtained by weighing freshly harvested cells. Dry weight avoids errors caused by endogenous water content (accumulated in the vacuole) and is more useful tool for biomass quantification than fresh weight.

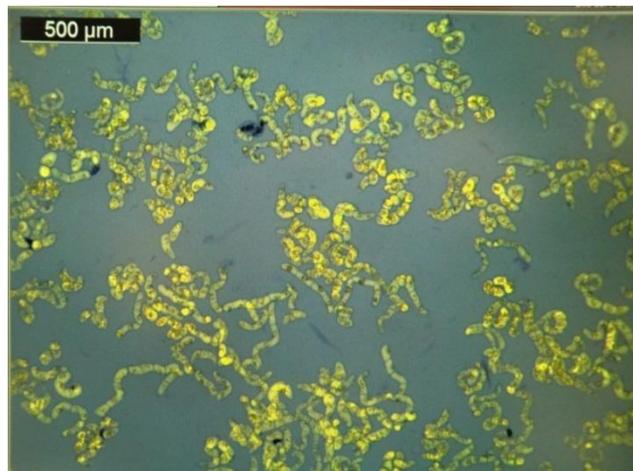


Figure 3: Cell viability

Source : Own.

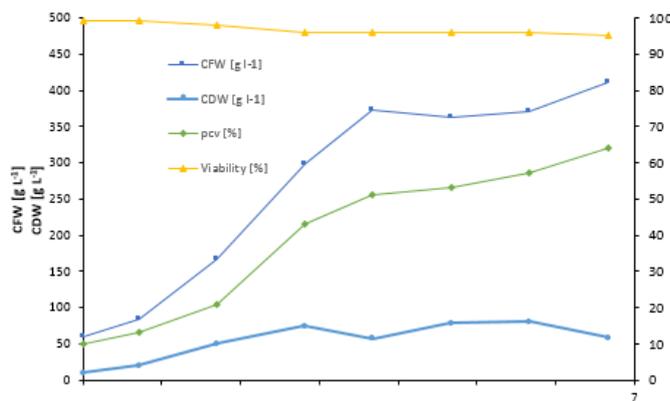
Dry weight measurement uses a known weight of fresh plant cells dried in an oven at a temperature between 50-60°C during 24 to 48 hours. Also fresh cells can be lyophilized.

In addition morphology and viability can be determined by microscope using Evan's blue, a dye which is used for staining suspended cell counts. Viability is defined as the ratio of viable cells to total cells and has a value between 0 and 100, described as 0% to 100%, blue cells are dead cells and the yellow ones are alive. [Figure 3]. Viability is a subjective determination, so two or three people are necessary.

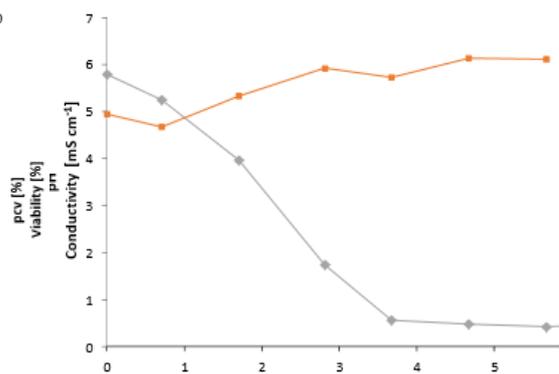
For cell suspensions like BY-2, another important parameter is determined, this parameter is called Packed Cell Volume or PCV, acquired by gentle centrifugation at low speed. PCV defines the ratio between the volume occupied by biomass to the volume of the whole sample (aliquots of 10 mL). Biomass and packed cell volume have

a correlation; also biomass can be measured by Neubauer type hemocytometer doing a manual counting.

Indirect monitoring is also a reliable measurement technique, consisting on measurements such as conductivity with a conductivity meter in liquid culture medium which allows the indirect monitoring of biomass growth. There exists an inverse correlation between electrical conductivity (expressed as mS cm^{-1}) and biomass. This can be explained by the uptake of nutrients and salts by the cells. Graphic 2 and 3 expresses this correlation.

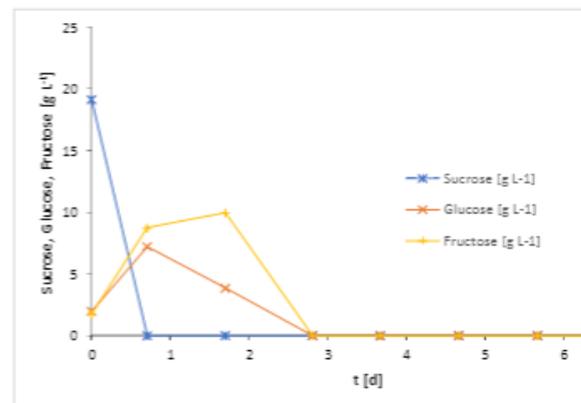


Graphic 2: Biomass increasing
Source : Own



Graphic 3: Conductivity decreasing
Source : Own

The pH measurement is routinely made during the cultivation of liquid cultures. A gradual drop in pH to a value around 4, reflects the initial ammonium uptake and acidification caused by cell lysis within 20-48 hours as is possible to see in graphic 3. The pH returns to a stable value of about 5 related to an uptake of nitrates after a few days of cultivation, finally at the end of the culture, medium reaches a pH above 6.



Graphic 4: Substrate
Source : Own

Metabolites are measured also, such as sucrose, glucose, fructose, ammonium, nitrate and phosphate. In graphic 4 it's possible to observe that sucrose is the first metabolite being consumed by the cells and after that it is observed the raises of glucose and fructose due to the fact that sucrose is metabolized in this two components.

Ammonium and phosphate are important metabolites due to the possibility of inhibition of the cell culture due to their amount.

2.2 Illumination

External illumination is an important parameter, with a range of 0-10klux it's possible to obtain a periodic cycle between 8h to 16 h or continuous illumination, by fluorescent lamps, light-emitting diodes like LEDs or Solid State Lamps (SSL). Furthermore, it's possible to use an internal illumination with encapsulated fluorescent lamps, and fiber optic cables.

This illumination is needed for certain kinds of cell cultures for grow or for elicitor but causes general problems such as heat development, and problems in the distribution of light intensity. In cosmetic industries, illumination is not IPC, this belongs to a dark cultures due to the low interest of the expression of chlorophyll which can dye with color green the product.

2.3. Types of bioreactors for plant cell suspension culture

For plant cell suspensions bioreactors can be divided into three main types according to their continuous phase: liquid phase bioreactors, gas-phase bioreactors and hybrid bioreactors.

Liquid-phase bioreactors:

Plants cells are immersed continuously and oxygen is usually supplied by bubbling air through the culture medium. **Mechanically driven bioreactor**, **pneumatically driven bioreactor** and **hydraulically driven bioreactor** belong to this category. Due to the low solubility of gases, the gas-exchange limitation and insufficient nutrient transfer, growth inhibition may occur in this kind of cultures.

Gas-phase bioreactors:

In these bioreactors, oxygen transfer limitation can be reduced or even eliminated, with bioreactors such as mist reactor or spray reactor. These reactors are specially manufactured for organ cultures like hairy roots, in addition a less hydrodynamic stress is acquired with this type of reactor.

Hybrid bioreactors:

With a combination of submerged and emerged bioreactors such as Wilson Bioreactor for hairy roots, the hybrid bioreactor switches from liquid-phase to gas-phase operation after the inoculation, distribution, attachment to immobilization points, and short growth phase of the cells.

An excellent plant cell growth with biomass productivity > 1g dry weight/L/day, requires an optimized and well characterized bioreactor configuration. Plant cells with high cell density and/or aggregate formation, as is the case of *Nicotiana tabacum*

BY-2 cell line, presuppose trouble-free inoculation, transfer and harvest, and consequently need specially designed bioreactor elements.

Mechanically or pneumatically driven aerated submerged bioreactors, are more often used in cell suspension cultures for large-scale rates. Derived from microbial fermenters stirred reactors, bubble column reactors and airlift reactors were initially used with only minor modifications to grow plant suspension cells.

In most plant cells cultivations, the air is directly introduced via a sparger (ring, pipe, plate, frit) positioned in the lower part of the bioreactor. Such direct aeration guarantees the highest possible aeration efficiency.

2.3.1 Re- and multi usable bioreactors for plant cell suspension culture:

Stirred

Independent of scale, production organism type, and product, stirred cell culture in which power input for mass and heat transfer is controlled mechanically, dominate. Since the end of 1950s: stirred bubble column and airlift bioreactor predominate over the other types of bioreactors, stirred dominate and are preferable for biomass productivity exceeding 30g dw L^{-1} .

This kind of bioreactors permits a maximum culture volume of 70m^3 with a $H:D= 2:2$ to $3:1$, often they are equipped with a bubble aeration, usually a sparger rings, with a $0.1\text{-}0.5$ vvm and a $k_L^*a \geq 10\text{ hr}^{-1}$.

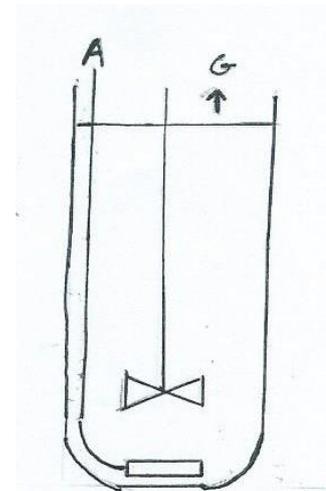


Figure 4: Stirred Bioreactor
Source : Own.

Large slow moving axial flow impellers with tip speeds up to 1.5 ms^{-1} such as marine impellers, special pitched blade impellers, spiral stirrers, helical ribbon impellers and anchor impellers. Also Rugston impellers suitable for limited applications or impellers with an improved design like concave blades.

Bubble columns and airlift reactors.

Pneumatically driven systems do not specifically require the use of immobilized cells as they were developed for free suspension cells. In bubble columns and airlift bioreactors, mass and heat transfers is mostly achieved by direct sparging of a tall column with air or gas that is injected by static gas distributors (diffuser stones, nozzles, perforated planes diffuser rings) or dynamic gas distributors such as slot nozzles, Venturi tubes, injectors or ejectors. While ascending gas bubbles cause random mixing in bubble columns, fluid circulation in airlift bioreactors is obtained by a closed liquid circulation loop, which permits highly efficient mass transfer and

improved flow and mixing. In airlift bioreactors, this circulation loop results from the mechanical separation of a channel for gas / liquid up flow and down flow.

Both airlift bioreactors with an external loop and with an internal loop are available.

Due to their relatively simple mechanical configuration bubble columns and airlift bioreactors are characterized by low cost in comparison with stirred bioreactors and also, the lower energy requirement, minimizes problems of scale-up. However this bioreactors have a potential disadvantages such as variations in biomass concentration, viscosity, surface tension, ionic concentration, inadequate mixing, foaming, flotation, and bubble coalescence.

With a maximum culture volume of 20 m³, and a H:D equal to 6:1 to 14:1, bubble columns take advantage in cultures which biomass productivities exceeds > 10 g dw L⁻¹. Bubble columns and airlift reactors are less often used than stirred bioreactors due to the limitations at high biomass productivities of heterogeneous distribution and oxygen transfer. In addition if high aeration rates are achieved with a sparger rings, extensive foaming flotation and wall growth phenomena may occurs. Moreover, bursting gas bubbles in bubble columns or in airlift bioreactors can raise the shear or hydrodynamic stress damage

Scientifics designed potential upscaling issues such as ceramic or sintered steel porous spargers, with an external aeration with a bubble-free aeration using an oxygen enrichment with only O₂ at lower aeration rates, and changes in geometry, an example of this change is in the case of balloon-type bubble bioreactors.

Hollow fiber

Hollow fiber bioreactors belongs to hydraulically driven devices, it reaches a high density with a tissue-like architecture. However hollow fiber reactors gain perfusion mod, this belongs to the hydraulically driven systems where energy input is produced by special double-phase pumps.

Cells grow in the extra-capillary space of thousands of fibers that have been potted into a cylindrical cultivation module. An oxygen enriched medium flows continuously through the fibers; this kind of bioreactor is often used by cell growth which produces secreted proteins. This bioreactor can be used by adherent or suspension cells, both are optimal for this device, however, besides probable mass transfer limitations there is a lack of data in process monitoring in the immediate cell environment. Also exist a risk of product contamination by cell fragments and cell lysis products as well as destruction of sensitive proteins by the high residence time of cells. But the main disadvantage of this bioreactor is their small culture volume, which ranges only from 2,5 to 1000L.

In conclusion, hollow fiber bioreactor is used for fast, flexible, small-scale production of antibodies for diagnostic and research.

Bed bioreactor

Also a hydraulically driven bioreactor, more directly linked to the use of cells for cultivation in an immobilized form. Cells are immobilized on microcarriers (small particles, usually spheres from 100 to 300 μm in size). Bed bioreactors can also be described in to main types, packed or fixed bed bioreactors and fluidized bed bioreactors.

The fixed bed bioreactors have a high density packet carrier material, which forms a fixed bed. This device is composed of cylindrical bioreactors chamber filled with carriers of porous glass or macroporous materials, a gas exchanger, a medium storage tank, and a pump that circulates the culture medium between the bioreactor and the medium storage tank. Severus disadvantages such as poor gas transfer and detachment, and cell washout limit the applications of this bioreactor, although have a low surface shear rate, absence of particle-particle abrasion and an increased space-time yield.

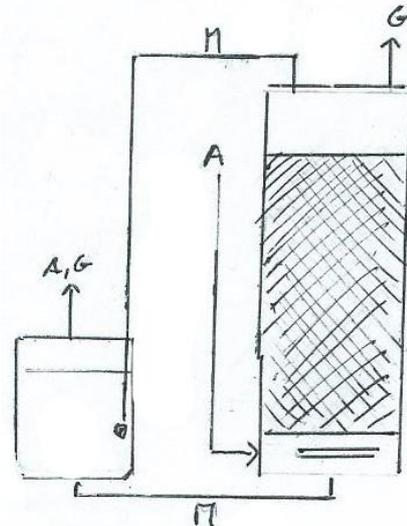


Figure 5: Bed bioreactor
Source : Own.

The packed bed bioreactor operates in up flow mode, the bed expands at high liquid flow rates and follows the motion of the microcarriers to which cells have been attached. To optimize the mass and heat transfer, fluidized bed bioreactors operation aims to provide a fluidized bed to ensure movement of all particles and avoid sedimentation or flotation.

Orbitally-shaken bioreactors:

Orbitally shaken single-use reactors are promising reactors, this affirmation it's possible due his characteristics, such as a simple and cost-efficient, because no complex built-in elements such as baffles or rotating stirrers are required. Also the liquid distribution induced by orbital shaking is well-defined and accurately predictable. And finally, the scale-up from small-scale systems, where shaken bioreactors are commonly applied, is simple and has been successfully proven up to the cubic meter scale. However they have some disadvantages, orbitally shaken single-use reactors are only suitable for certain applications such as cultivating animal or plant cells with low oxygen demand. [5]

Also orbitally shaken bioreactors can be performed in single-use conformation

2.3.2 Single-use and disposable bioreactors for plant cells and tissue cultures

Cultivation bags or rigid cultivation containers for single-use are used in these bioreactors. They are manufactured from different kinds of polymeric materials and they must be sterilized by gamma radiation, customized and validated.

Different types of bioreactors are suitable for plant cells cultures and root cultures such as mechanically driven and pneumatically driven. Mechanically driven like orbitally shaken, wave-mixed bioreactors, stirred bioreactor, bioreactor with vertically oscillating perforated disk are different systems for these kind of bioreactors. Instead of the large number of mechanically bioreactors pneumatically bioreactors only exist in driven bubble columns.

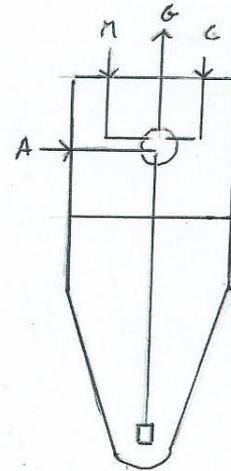


Figure 6: Single-use bioreactor
Source : Own.

Life-reactor (Osmotek)

Is the first disposable bioreactor for plants cells described in literature; with a capacity of 1.5 to 5 liters; it is a pneumatically driven bubble column. Used successfully for production of embriocrops such as potatoes, bananas, ferns and gladiolas.

Plastic-lined Bioreactor

A bubble column integrated in a plastic bag was the basic idea for the Life bioreactor, with a capacity between 28,5 to 100 liters, which is a pneumatically driven bubble column.

Wave-mixed bioreactors

Mechanically driven bioreactor with a capacity between 1L to 500L, is a system for different vendors, and they have differences in rocking motion (1D,2D or 3D), bag geometry and size instrumentation. These reactors have a plastic disposable cultivation chamber; the gas-permeable and surface-aerated bag is fixed by a clamp arrangement and moved on the rocker unit. Mass and energy transfer is manually adjusted via the rocking angle, rocking rate, and filling level. They present multiple advantages such as bubble- free surface aeration, well-investigated, uniformity of energy dissipation and negligible foaming, this means a low shear stress and an increase of oxygen transfer. This bioreactor is consequently a suitable bioreactor for cell growth, in contrast, they have some problems with rheological issues of plant cultures.

Commercial examples: Wave Bioreactor system 500/1000, AppliFlex, Cell-tainer single-use bioreactor, BIOSTAT® CultiBag RM, Tsunami Bioreactor.

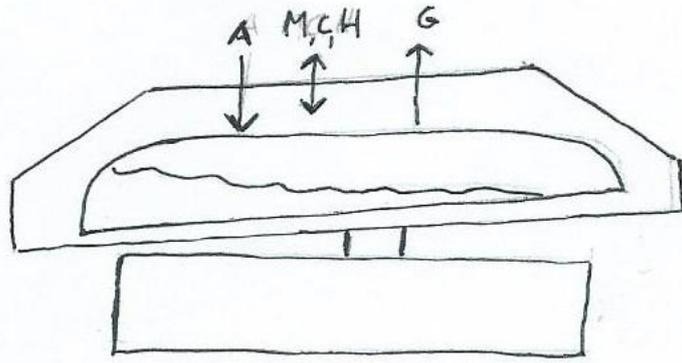


Figure 7: Wave bioreactor
Source : Own.

- The BIOSTAT® RM
A fully GMP compliant, single-use, wave-mixed bioreactor and single use Flexsafe® bags are proven for a broad range of different cell lines incl. CHO, NS0, SF9, E.coli and mesenchymal stem cells. It takes the advantage of an excellent cell growth and robustness, high type of supply and consistent quality and easy to use rocker with advanced control capabilities. [6]
- AppliFlex®
Designed by Applikon® biotechnology, the Appliflex® bioreactor consists of a 10-liter, 20-liter and 50-liter bioreactor bag designed for single-use. It's especially manufactured for mammal cells like CHO and for cell lines of insects like sf-9 and sf-12. [7]

Other bioreactor developed and tested by Nestle are also a suitable instrumentation for plant cell and tissue cultures, such as Wave and Undertow Bioreactor (WUB) a mechanically driven layer with a capacity between 10 to 100 L with only a mobile part which gets up and down in order to make waves. Slug Bubble Bioreactor (SBB), a pneumatically driven with a capacity of 10 to 70 L, with a slug flow regime but this bioreactor has a handicap: the bubble flow is not homogeneous. Finally also developed by Nestle exist the Simple immersion bioreactor Box-in-bag Temporary Immersion Bioreactor.

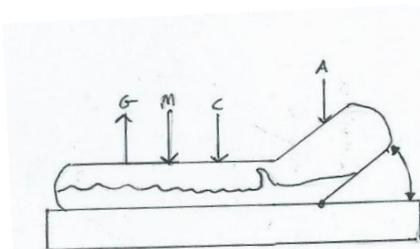


Figure 8: WUB
Source : Own.

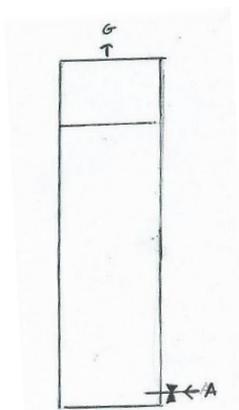


Figure 9: SBB
Source : Own.

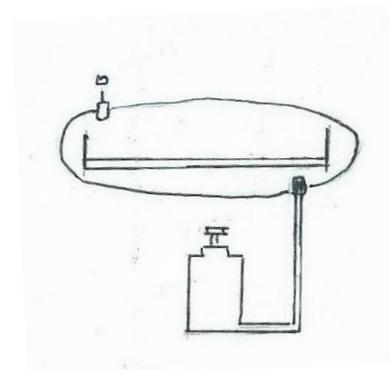


Figure 10: Simple immersion bioreactor
Source : Own.

Protalix Bioreactor:

It is a pneumatically driven bubble column bioreactor with a high capacity over than 400 L, restricted by GMP-production.

Large-Scale Disposable Shaking Bioreactors:

These bioreactors are cylindrical vessels of 20 L and 50 L mounted on a standard RC-6 shaking machine). Advantages of this single-use bioreactor over others disposable devices: it's easy to use, have a well-defined gas/liquid mass transfer area , a low levels of hydromechanical stresses due to homogeneous distribution of the power consumption and most important, the reduction of initial costs (no need to purchase special bags and rocking machines)

Orbitally-shaken bioreactors:

Defined at Re- and multi usable bioreactors

2.3.3 Re- and multi usable bioreactors for root culture:

Liquid-phase, gas-phase and hybrid bioreactor systems are all suitable for growing root cultures: bubble columns, airlift bioreactors and mist bioreactors with sterile baskets installed. They have a maximum culture volume of 10 m³ (balloon type bubble bioreactors).

On the other hand, cultivation of roots has some difficulties such as varying root thickness, root length, number of root hairs and different root branching sequence that produce a non-homogenous growth and production. At the same time these cultures have tendency to form clumps inherently composed of primary roots and their bridged lateral roots. Root has been more damaged by shear stress so mostly of this cultures need an isolation of the roots from the impeller.

This kind of cultures must be immobilized, which promotes root growth, this is acquired with horizontal or vertical meshes and cages or with polyurethane foam.

Mist reactor

Mist bioreactor is a gas-phase instrument with the highest potential for the cultivation of hairy roots in gas-phase bioreactors. With a droplet generator the roots have an optimal growth if critical droplet size is between 1µm and 35 µm since in this case liquid nutrients are homogeneously distributed and the gas transfer into the rots is free of limitations such as oxygen stress. The

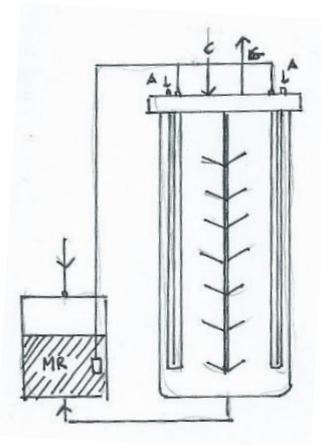


Figure 11: Mist bioreactor
Source : Own.

cultivation container has an immobilization support with horizontal and vertical meshes.

Low Cost Mist Bioreactor (LCMBs)

Is the largest gas-phase bioreactor with a capacity of 60L. Low cost mist bioreactors were designed to grow *Artemisia annua* transformed roots and *Dianthus caryophyllus* shoots. The reactors use similar mist generators but the culture chambers were modified to meet the requirements of each application. [8]

Wilson bioreactor

Is the largest hybrid bioreactor system with a capacity of 500L. The spray reactor reminds a gas-phase bioreactor with a cultivation container with horizontal meshes. This bioreactor has some problems such as very laborious handling and often associated with contamination issues, now is no longer in use.

Balloon-type

Is a spherical-glass bioreactor, which is used for root culture due their advantages of easy moisture and simplicity, although this assets, are no-longer in use due to easy contamination and difficulty of take out the roots. Useful for extracellular products.

2.3.4 Single use vs re- and multiusable bioreactors. Advantages and disadvantages.

In R+D biofactories have different trends than commercial industries, they need a scale-down bioreactors in a small scale laboratory, multifermenters systems equipped with stirred bioreactors or bubble columns which acquires a high amount of data in less time, such as bench top bioreactors with industrial control mode, low cost and disposable bioreactors.

Disposable or single-use bioreactors have different advantages over typical bioreactors, such as short time for production, lower costs, and high safety. For these reasons these bioreactors are used in the emergent industry. Furthermore a high flexibility and simplicity also sterilization is not needed, neither cleaning times.

Although, single-use bioreactors are well established, not always are optimal, they have some weakness such as limited scalability, limited standardization, an increased storage requirements and increase of waste generation (plastic bags have to be throw away) also a repetitive costs and security risks at supply the bioreactors with the mixture of culture and substrate.

Now, some possible threats to increase production and acquire low costs and defeat the supplier dependence are possible with an union with the supplier; or with improved films as the reduction of Irgafos 168 in the new improved films.

* IRGAFOS 168 is a hydrolytically stable phosphate processing stabilizer. As a secondary antioxidant, IRGAFOS 168 reacts during processing with hydroperoxides formed by autoxidation of polymers preventing process induced degradation and extending the performance of primary antioxidants. [9]

2.4 Summary table

Re-usable						
Type	Dirven	Aeration	Advantages	Disadvantages	Uses	
Stirred	Mechanic	Bubbles/airlift	-Easy scale up -Simple -Well-known	-Shear stress -Mechanic stress -Foam	Suspensions	
Bubble-column Airlift	Pneumatically	Bubbles/airlift	-High aeration -High efficient mass transfer -Low cost	-Foam -Shear stress -Lack of homogeny -Viscosity -Inadequate mixing	Immobilized Suspension	
Hollow fiber	Pneumatically	Perfusion pumps	- Easy downstream - Fast - Flexible - Small-scale	- Mass transfer limitation - Lack of data monitoring - Contamination - Destruction of cells - Low culture volume	Adherent cells Suspended cells proteins	
Bed bioreactor					Immobilized cells	
	Hydraulic	Perfusion pumps	-High density -Low surface shear	-Poor gas transfer - Difficulties at downstream - Increased space-time yield		
	Hydraulic	Perfusion pumps	-Optimized mass transfer -Avoid sedimentation	-Poor gas transfer -Difficulties at downstream		
Orbitally	Mechanic	Bubbles/airlift Absence	-Simple -Well cost-efficiency -Well defined liquid distribution -Easy to scale-up	-Only suitable for certain cell lines	Low-oxygen cell lines	

Single-use Type	Dirven	Aeration	Advantages	Disadvantages	Uses
Stirred	Mechanic	Bubbles	-Easy scale up -Simple -Well-known	-Shear stress -Mechanic stress -Foam	Suspensions
Life-reactor	Pneumatically	Bubbles	--Easy scale up -Simple -Well-known	- Low culture volume	Embriocrops
Plastic Lined	Pneumatically	Bubbles	--Easy scale up -Simple -Well-known	- Mass transfer limitation - Lack of data monitoring - Contamination - Destruction of cells - Low culture volume	Adherent cells Suspended cells
Wave	Mechanic	Bubbles	Bubble free surface aeration Well-investigated Uniformity of energy Negligible foaming Low shear stress Increase of oxygen transfer	-Rheological issues of plant cultures.	Suspension cells Immobilized cells
Shaking	Mechanic	Bubbles Absence	-Easy scale up -Simple -Well-known Low mechanical shear Low cost	-Only suitable for certain cell lines	Adherent cells Suspended cells
Orbitally	Mechanic	Bubbles Absence	-Simple -Well cost-efficiency -Well defined liquid distribution -Easy to scale-up	-Only suitable for certain cell lines	Low-oxygen cell lines

3. Suitable bioreactors for *Nicotiana tabacum* BY-2

3.1 *Nicotiana tabacum*

Nicotiana tabacum is a stout annual of the tomentosae family, approx 1–3 m high. The stem is erect with few branches. Leaves are ovate, elliptic, or lanceolate, up to 100 cm or more in length, usually sessile or sometimes petiolate with frilled wing or auricle. The inflorescence is a panicle with distinct rachis and several compounds branches. Flowers are light red, light pink, or white. The fruit is a capsule approx 15– 20 mm long, narrowly elliptic ovoid or orbicular. Seeds spherical or broadly elliptic, 0.5 mm long, brown, with fluted ridges. [10]

It's originated from the borders of Argentina and Bolivia. It has been cultivated in pre-Columbian times in the West Indies, Mexico, Central America, and the northern region of South America. It is now found worldwide as a cultivated crop. [10]

It have different traditional medical uses such as in East Africa whom use this plant in order to destroy worms in sores, in Tanzania they use for stimulate the labor of child birth and in Haiti it used for a cure of pneumonia and bronchitis. [10]

3.2 Tobacco Bright Yellow-2 BY-2

After more than 20 years of proof-of-concept research, the manufacture of biopharmaceutical proteins using plant cell suspension cultures was finally approved by the Food and Drug Administration (FDA) in May 2012. The regulators agreed that Eleyso, a recombinant human glucocerebrosidase indicated for the treatment of Gaucher's disease, could be marketed for therapeutic use in humans [11]



Figure 12: BY-2 cells
Source : Own.

First of all plantibodies have to be described:

plantibodies are immunoglobulins produced in intact genetically modified plants or plant cells. The growing need for antibody products in diagnostic and therapeutic applications explains the strong interest in alternative safe cost-efficient expression systems.

Since antibodies are specific glycoproteins, the comparison of plant cells to other expression systems concerning glycosylation is of high interest. This comparison displays that recombinant, plant-produced glycoproteins have much in common with human N-glycan structures in regards to posttranslational modification rather than their microbial counterparts. In addition, it is considered that, using plant cells for

recombinant protein production enables the reduction of health risks that arise from contamination with human pathogens or toxins.

One of the most important Tobacco Bright Yellow-2 (BY-2) cell lines secrete a human antibody called IgG1 antibody M12, a tumor specific antibody. [12]The BY-2 cell culture was established by callus induction from a seedling of *Nicotiana tabacum* L. 'Bright Yellow 2' (tobacco) in Japan in the 1970s [3]

BY-2 suspension cells are a widely used biological material for studying plant cell morphology and physiology. These cells are easy to transform and maintain in culture and tolerate transformation with fluorescent proteins such as the green fluorescent protein and its derivatives. These, by the addition of plant or mammalian targeting sequences, can be directed to specific subcellular locations for the study of cell dynamics in vivo.

These cells have the advantage over other cells that are well established, have a simple transformation, with *Agrobacterium*, and regeneration to new plants. At the same time present easy handling and subcultivation cycles and favorable growth characteristics, with a doubling time between 13 to 24 h. Finally, this cell line can be cultured in bioreactors of up to cubic meter scale. [13]

The cultivation of BY-2 cells in disposable bioreactors is a useful alternative to conventional stainless steel stirred-tank reactors, and orbitally-shaken bioreactors could provide further advantages such as simple bag geometry, scalability and predictable process settings [3]. The culture of these cells permits to shift away from stainless steel bioreactors towards disposable and pre-validated plastic alternatives, which still enable strict process control [7]

3.3 Transformation of BY-2 cells

The transformation of BY-2 cells of *Nicotiana tabacum* uses *Agrobacterium*, for this procedure, it's necessary to start a new subculture three days before the transformation.

First of all it's imperative to grow a culture of *Agrobacterium tumefaciens* with a binary vector containing the appropriate construct, after this it's possible to establish a stable transformation of BY-2 cells. It's possible to use a wild type or a BY-2 yet transformed for co-expressions. [14]

Following the protocol the first step is the preparation of inoculum of agrobacteria, inoculates to a BY-2 suspension, incubation, transfer in a petri dish with BY-2 medium, suspend wash and transfer and finally screen the callus in order to search fluorescent transformed cells. Before more downstream cells need to be subcultivated for 4 weeks. If it's necessary to use a co-transformation start the second transformation

once stably expressing of the first one are established. All the protocol of transformation could have seen in the Annex. [15]

3.4 Transformation of BY-2 M12

Using the same protocol of transformation of BY-2 cells it's possible to establish a well-defined cell line of M12, but it's necessary to use protoplasts of BY-2, protoplasts are cells that had his cell wall removed. It's necessary to prepare a suspension, for determine the protoplast viability it's possible to dye them with fluorescents dyes. After ,a flow-cytometry sorting determine wild type protoplast, size and granularity, a flow-cytometry sorting consist in individual cells which are simultaneously analysed by passing through a laser beam at a very high rate, flow cytometry is a unique tool to analyse the characteristics of cell populations on a single-cell basis rather than on a population average. [16]

For BY -2 citometry uses a Fluorescence-Activated Cell Sorting (FACS). FACS was based on the assumption that the amount of the fluorescent marker protein correlates with the amount of antibody produced within a cell allows the rapid identification and isolation of the most productive cells from within a heterogeneous. [17]

BY-2 cells were transformed with plant expression vectors encoding either the M12 antibody in combination with DsRed or the fluorescent protein tGFP so BY-2 cells co-produce the human antibody M12 and the selectable marker protein DsRed; the coding sequences of the recombinant proteins. [16]

3.5 BY-2 medium

Before start a discussion of which bioreactor is more suitable for BY-2 cultures, it's basic to know the components of the medium and the most important parameters, such as aeration or pH, in order to start a culture of this cell line.

The culture Medium is a modified MS (after Nagato 1992, it have MS salts, thiamine + myo-inositol), Miller's, 2,4-D hormone and sucrose. The necessary pH is 5.6 and it's possible to establish by adding KOH. If it's indispensable to establish a solid culture add phytigel. If antibiotics for selection have to be added, it's possible to add carbenicillin to kill bacteria or kanamycin to select for transformed BY-2,

In the laboratory, growth liquid can be put on an orbital shaker at 120 rpm at 27°C in the dark, or a callus at 27°C also in dark. Cultures liquid has to be transferred every week and callus every three weeks. All the components with the volumes are in Annex 2 [18]

3.6 Culture conditions:

A design of a bioreactor usually ensures cell growth without cell damage, however, problems can arise from the complex rheological characteristics of plant cell suspensions since they become non-Newtonian fluids when they are growing and consequently, viscosity rises. [19]

In addition, possible foam formation, bursting bubbles and shear stress can reduce the production.

As is said before, pneumatically driven bioreactors have a serious problem of foaming consequence of his bubble columns, this kind of bioreactors only mixes with bubbles, that give a huge amount of problems such as non-homogeneous mixing, and shear stress with bubble rising and bursting, therefore pneumatically driven bioreactors could be discarded for *Nicotiana tabacum* BY-2 cells.

3.7 Culture studies

In consequence of the lack of information about cultures of M12 and used bioreactors, this area it's divided by two terms, production of M12 and general parameters, in contrast, all the bioreactors are compared by production of recombinant proteins, pcv or fresh weight.

3.7.1 M12

Eibl, exposes an overview of the bioreactors used for recombinant protein production with plant cell suspension cultures, where it is shown that for *Nicotiana tabacum* cultures stirred bioreactors are predominant; the possible explain of this huge amount of bioreactors is an easy scale-up of this devices. Also WUB bioreactors are used. In a summary of growth and M12 production studies with single-use bioreactors, it seems that BioWave (Initial volume: 1L) has a maximum growth rate, and a maximum M12 accumulation per volume (1,6 mg/L) over the others single-use bioreactors. In the other hand maximum produced biomass (pcv) owns AppliFlex (Initial volume: 5L/ inoculum of 15%) 70% of pcv and cultiflask 50 (Initial volume of the inoculum: 10mL) an 80%, beginning in all the cases with a 10% inoculum. In addition they tested a S.U.B (Initial volume: 25L/ inoculum of 15%) Also in wave-mixer, has lower foaming and it's not necessary to add antifoam agents. [3]

Raven, with an inoculum of 5 % uses a different bioreactors types in order to determine the possibility of scale-up of each device. Shake flasks with the BPM-60 system(Initial volume: 50mL), orbitally shaken SB200-X (Initial volume: 5L) are used in this study, with a results of 62% of pcv for shake flasks and 68% for orbitally, fresh weight differs more, with a 387g/L for orbitally and 300g/L for shake. Finally product titer of 20mg/L in shake and 18mg/L in orbitally. [12]

In addition she investigated the effects of inoculum density on the cultivation time and it seems that a inoculum of 8% have more harvest cells than the inoculum of 10% or 5%

My own work was to study the cell growth and human antibody production with BY-2 suspension cells in the wave-mixed BIOSTAT® RM operating with CultiBag RM 2L, with a cell line able to express tumor-specific human IgG antibody M12. An inoculum of 10%, gave the following results: 47% of pcv and a CFW of 3,2 g/L, also a low titer of protein expression, only 8.5 µg /L, but calculated concentrations over the process time did not follow a saturated line, so the values are not to be trustable as is show in figure 13.

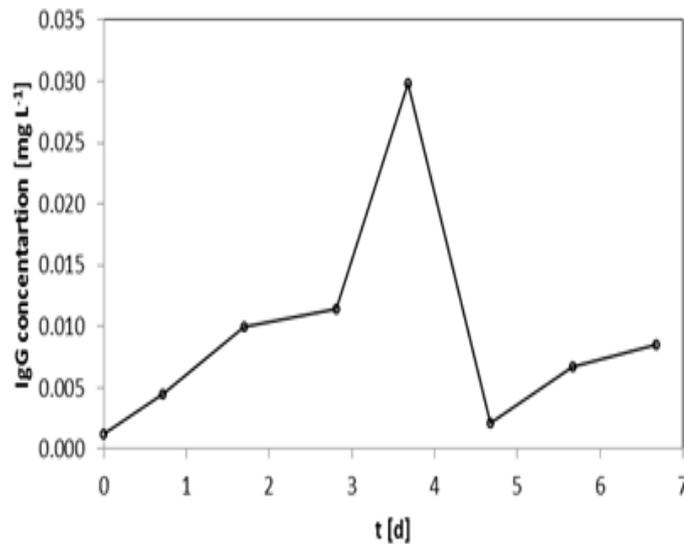


Figure 13 Human IgG M12 expression of BY-2 suspension cells
Source : Own.

3.7.2 BY-2 general parameters

Lauri, uses stirred bioreactors and t-flasks in order to show a possible scale-up in BY-2. If fresh weight and dry weight in g/L of the different bioreactors are compared, it shows that stirred bioreactor has bigger amount of biomass than Shake flasks. With a fresh weight of a 650 g/L and a dry weight of 47 g/L in the last day in stirred bioreactor in comparison to 200g/L FW and 17 g/L DW obtained in shake flasks. Initial volume is not said. [20]

Imseng, describes the mass propagation of *Nicotiana tabacum* cv. BY-2 suspension cells in the Finesse SmartRocker® bioreactor (wave mixer) at laboratory scale. With a starter volume of 1L of the inoculum, it achieves maximum fresh cell weights of about 351±9 g·L⁻¹, corresponding to packed cell volumes of 74±2 %, which shows the applicability of the Finesse SmartRocker® (wave) bioreactor to cultivate fast growing plant suspension cells. But are lower than values reported by Kirchhoff (2012) for the Applikon® 3L(Stirred) stirred bioreactor (fresh weight 412 g·L⁻¹) Even higher fresh biomass concentrations of up to 470 g·L⁻¹ and 95 % pcv have been achieved in the Finesse SmartGlass® 3L bioreactor (Finesse Solutions, Inc.) [21]

3.8 Summary table

Inoculum 10%					
Bioractor	Product	Pcv %	Fresh weight	Starter Volume	Reference
Wave	1,6 mg/L	63		1L	[3]
Orbitally Cultiflask 50	2.2mg/L	80		10mL	[3]
Wave	8.5 µg /L	47%	3,2g/L	1L	Own
Inoculum of 15%					
S.U.B	Non detected	52		25L	[3]
Wave AppliFlex	Non detected	70		5L	[3]
Inoculum of 5%					
Orbitally	18mg/L	68	387g/L	5L	[12]
Shaken	20mg/L	62	300g/L	50mL	[12]
No inoculum information					
Wave		74±2	351±9 g·L-1	1L	[21]
Stirred		95	470 g·L-1		[21]
Stirred			412 g·L-1		[21]
Stirred			650 g/L	NS	[20]
Shake			200g/L	NS	[20]

4. Discussion

Plant cell cultures have plenty advantages in pharmaceutical industries and in cosmetic ones too, not only have advantage over traditional wild-plant harvesting, they are over bacteria cultures due their post-transcriptional capacity, better than insect cells because of their well-known metabolic pathways and superior than mammal cells due to their safety (no human viruses can sprout) and low cost.

As is said scientists well-know about their mediums, cultures, characteristics, and critical points of culture, modes and how to transform cells to more efficient ones. Now the main problem is the scale-up of our bioreactors, due the simplicity, low cost and safety pharmaceutical and cosmetic industries have an eye on this type of manufacture of new products, so bioreactors have to be improved.

After all the bibliographic research and thanks to summary tables of point 2.4, it's possible to determine the pros and cons of each bioreactor quickly.

In the early past, industries uses stirred bioreactors, hollow fibers and bubble columns for their production as it's possible to see in Phyton Biotech™ [22], which uses bioreactors of stainless steel. But now, single-use bioreactors are gone to set aside this last devices, PhytoCellTec™ [23] (MibelleGroup) uses *Malus Domestica* for obtaining a component for a liposomal active ingredient based on stem cells from the Uttwiler Spätlauber apple, this cosmetic industry uses biowave bioreactors in sequence instead of huge stainless steel bioreactors. Single-use bioreactors are a reality in R+D laboratories due to their high amount of data in less time, low cost and safety but in industry stainless steel bioreactors are yet implemented; however low-cost safety, short time of production(it's not necessary to get clean and disinfected), high flexibility and simplicity are going to change this trend.

Products as their culture types are different, consequently the disposable bioreactors are lots, it seems that stirred bioreactors with different impellers are the most used kind of device, it has plenty of advantages and low cons, so the combination of stirred devices with bubble columns or Airlifts, suggests a great solution. Orbitally bioreactors have more advantages than stirred but they only can be used for low-oxygen cell lines. Hydraulic and hollow fibers are only focused in Immobilized and attached cells.

Root culture, due their difficulty and own characteristics has specific bioreactors, now the most used is the Mist bioreactor, Wilson bioreactor have a great capacity and product titer but it has plenty of contamination.

In the other hand, single use bioreactors, stirred ones have importance, but their lack in no foam formation and shear stress improves the acceptance of wave-mixing

bioreactors, the only problem of this kind of device is the rheological issues of plant cultures, but this could be avoided by rise the rocking angle and velocity.

Finally, the hypothesis of the most suitable bioreactor for By-2 it was not confirmed nor rejected due the lack of data, nothing has yet published on the cultivation of BY-2 suspensions cells in commercial available orbitally, bag or disposable bioreactors [3]. But using summary table 3.8, it seems that stirred bioreactors have more fresh weight and high pcv, using different inoculums and starter conditions (only differs in starting volume). Furthermore it could we assumed that orbitally shake are better than shaking ones, this is owing to the mechanical stress provided by the impact with plastic/glass of the bioreactor

With my personal experience with this kind of devices, I have to say that they are not very trustable, the pcv, fresh weight can differ in large proportion, so it's necessary a well-established protocols and experience.

For reach a conclusion of the hypothesis of this study I would use different bioreactors in sequence (at least three of each type) with the same inoculum and starting volume, so for a denouement, wave-mixer are theoreticaly better to the other devices but in practice it's not clear.

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ANNEX

Stable transformation of BY-2 cells

Introduction

- Stably transformed BY-2 cells are generated by co-cultivation with *Agrobacterium tumefaciens* (strains GV3101, LBA4404 or EHA105)
- Adapted from: Graumann K and Evans DE, (2014), "Dynamics of the Plant Nuclear Envelope during Cell Division", Methods in Molecular Biology

Materials

1. LB medium with and without appropriate filter-sterilized bacterial selection antibiotic
2. Acetosyringone (3',5'-Dimethoxy-4'-hydroxyacetophenone) as 100 mM stock in ethanol
3. Sterile antibiotic stock solutions

Timentin (ticarcillin disodium/ potassium clavulanate) and carbenicillin should be used to kill agrobacteria after co-cultivation. The working concentration of these antibiotics is as follows: 20 mg/l timentin, 100 mg/l carbenicillin. Antibiotics are also required for selection of transformants. The choice of the antibiotics is dependent on the vector used but can include kanamycin and hygromycin B. Working concentrations for these two are as follows: 100 mg/l kanamycin, 40 mg/l hygromycin B.

4. *Agrobacterium tumefaciens* strains GV3101 or EHA105 or LBA4404 transformed with vector containing appropriate construct should be used.
5. BY-2 suspension culture (3-day-old; prepared as described above)
6. Sterile liquid BY-2 medium (approximately 70 ml per construct)
7. Sterile solid BY-2 medium plates without antibiotics (1 plate per construct) and plates with selection antibiotic, 100 mg/l carbenicillin and 20mg/l timentin (10 plates per construct)
8. Stationary incubator at 25oC, no light
9. Shaking incubator, 25oC set to 130 rpm, no light
10. Sterile pipette tips and pipettes
11. Sterile 1.5 ml microcentrifuge tubes (1 per construct) and 15ml sterile centrifuge tube (2 per construct)
12. Forceps, sterile
13. Laminar flow hood
14. Tin foil
15. Parafilm
16. Benchtop microcentrifuge
17. Coldroom or refrigerator

Method

Growing agrobacteria

Inoculate 5 ml LB medium (containing appropriate filter-sterilized bacterial selection antibiotic) with a single colony of *Agrobacterium tumefaciens* GV3101 or EHA105 or LBA4404 strain transformed with a binary vector containing the appropriate construct. Alternatively, use swab of glycerol stock for inoculation. Incubate 16 to 20 h at 180 rpm and 28°C.

Stable transformation of BY-2 cells

Use a 3 d old BY-2 suspension culture (either wild type for single transformation or transformed line for co-expression)

Prepare agrobacteria as follows:

1. Centrifuge 1 ml liquid overnight agrobacteria cultures at 5 000 *g* for 5 min at RT. In a laminar flow hood or by the flame, remove supernatant and resuspend cells in 1 ml LB medium containing acetosyringone (add 20 μ l acetosyringone stock to 10 ml medium). Repeat centrifugation to finish first wash step. Repeat wash step two more times. After third wash, resuspend cells in 1 ml LB medium containing acetosyringone and incubate for 1 h at 4°C.
2. In a laminar flow cabinet, transfer 7 ml of the 3 d BY-2 culture to a 15 ml sterile tube, add 1.2 μ l acetosyringone and 100 μ l of prepared agrobacteria. Gently invert tube several times to mix cultures and then pour onto plate containing solid BY-2 medium with no antibiotics.
3. Seal plate with micropore tape (SLS) and either wrap in aluminium foil or place in a blacked out box, and incubate for 3 d at 25°C in the dark without shaking
4. After incubation, in laminar flow hood, transfer BY-2 cells from plate for washing by gently tapping the petri dish to loosen cells and then rinsing cells off the plate with 5-10 ml liquid BY-2 medium; transfer to sterile 15ml tube.
5. Wash the BY-2 cells three times with 15 ml liquid BY-2 medium containing 100 mg/L carbenicillin and 20 mg/L timentin; for each wash step, centrifuge the cells at 3000 rpm for 5 min (set breaks and acceleration to 0), remove supernatant and resuspend in approximately 10 ml medium (final volume 15 ml). Alternatively to centrifugation, cells can be left for 10 min to settle

naturally before removing supernatant. However, centrifugation causes fewer cells to be lost.

6. After the final wash step resuspend cells to a total of 10 ml. Transfer 1 ml of resuspended cells onto a plate containing solid BY-2 medium, timentin, carbenicillin and appropriate selection antibiotic.
7. Gently rotate plate to spread cells over the surface of the solid medium.
8. Seal plate with Parafilm and incubate in the dark at 25°C without shaking for 4-6 weeks until calli appear.
9. If constructs are fluorescent, use fluorescence stereomicroscope to screen the calli. Transfer calli to suspension as detailed in method for producing BY-2 suspension culture.
10. Subculture cells for at least 4 weeks before other downstream applications.
11. For double-transformation, BY-2 cells are first transformed with one construct and, once stably expressing suspension cells are established, these may be used for a second transformation with the other construct.

Maintenance of BY-2 cell cultures

Culture Medium

Modified MS (Nagato 1992)

Per liter

- 4.3 g MS salts (Sigma M5524) (in fridge)
- 10 ml thiamine (0.1 g/l) + myo-inositol (10 g/l)
- 3.5 ml Miller's (60 g/l KH₂PO₄)
- 2 ml 2,4-D (100 mg/l)
- 30 g sucrose
- pH 5.6 with KOH

Autoclave and store in airtight bottle for up to a month or so

For plates: add 1.2 g phytigel per 0.5 l

Antibiotics for selection (for example) 500 µg/ml carbenicillin (to kill bacteria, stock is 250 mg/ml in H₂O) 50 µg/ml kanamycin (to select for transformed BY-2, stock is 50 mg/ml in H₂O)

Growth

Liquid: on orbital shaker (120 rpm) at 27°C in the dark

Callus: at 27°C in the dark

Transfer Cultures

Liquid: EVERY WEEK, 1:50 (i.e. 400 µl per 20 ml MS) keep old cultures around for a week on RT shaker as backups

Callus: EVERY 3 WEEKS, wrap with parafilm and keep in airtight box in incubator (to prevent drying out)