REVIEW

Bioreactor technology for clonal propagation of plants and metabolite production

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Abstract Plant cell culture in bioreactors is an enabling tool for large scale production of clonal elite plants in agriculture, horticulture, forestry, pharmaceutical sectors, and for biofuel production. Advantages of bioreactors for plant cell culture have resulted in various types of bioreactors differing in design, operating technologies, instrumentations, and construction of culture vessels. In this review, different types of bioreactors for clonal propagation of plants and secondary metabolites production are discussed. Mechanical and biochemical parameters associated with bioreactor design, such as aeration, flow rate, mixing, dissolved oxygen, composition of built-up gas in the headspace, and pH of the medium, are pivotal for cell morphology, growth, and development of cells within tissues, embryos, and organs. The differences in such parameters for different bioreactor designs are described here, and correlated to the plant materials that have been successfully cultured in different types of bioreactors.

Keywords bioreactor types, mechanical and biochemical parameters, plant cell culture, plant clonal propagation, secondary metabolite production

Introduction

Large scale production of high value plants for agricultural applications, biofuel production, ornamental purposes, or molecular pharming within the pharmaceutical sector relies on production of clonal propagules from selected superior genotypes. Clonal propagation is also a prerequisite for production of plants that have been genetically improved by traditional breeding strategies, or direct genetic transformation. Different clonal propagation techniques are utilized for production of clonal plant propagules depending on the optimal technique for the plant species of interest and the ultimate use of the propagules. For example, meristem culture or auxiliary bud/shoot culture is used for propagation of many important species of ornamental plants (Rout et al., 2006) and medicinal plants (Rout et al., 2000). For some agricultural plant species, normal looking clonal plants cannot be obtained from meristems or bud/shoot explants. However, in some cases the process of somatic embryogenesis is applicable. Somatic embryogenesis is a prospective clonal propagation technology for several plant species. Many of the *in vitro* metods in use for clonal propagation of plants or plant propagules for production of plant biomass, metabolites, or enzyme have been shown to be most effective when carried out using bioreactors.

Bioreactors are closed, sterile systems for clonal propagation of organogenic propagules or somatic embryos. The internal environment of bioreactors is typically controlled to different extents depending on the model of bioreactor and plant material. The physical and chemical parameters associated with the bioreactor environment are medium circulation, mixing, aeration, cell suspension, temperature, pH, and dissolved oxygen. Different plant species and propagation materials have different requirements that will dictate the specific settings for internal bioreactor environment and determine the most appropriate bioreactor model to be used.

Growth and development of plant cells *in vitro* mostly depend on liquid medium circulation, mixing, and aeration

for distribution of oxygen and nutrients (Illing and Harrison, 1999; Curtis, 2005). So an effective design of a bioreactor must ensure this growth favorable environment. Bioreactor design is technically challenged by the properties of suspension culture, such as viscosity, shear sensitivity of cells, tendency of cell aggregation and foam formation (Doran, 1999). Especially the shear sensitivity of cells plays a significant role, because plant cells have a low shear tolerance (Mandels, 1972). This leads to several novel designs of bioreactors which have become successful in micropropagation. Some of them have also been commercialized. The main difference among these bioreactors is their means of operation. Therefore bioreactors used for clonal propagation and metabolite production can be divided into two broad categories, (i) mechanically operated bioreactors and (ii) pneumatically operated bioreactors. Stirred tank, rotating drum, and spin-filter bioreactors fall into the first category. The second category bioreactors are comprised of bubble column bioreactors, airlift bioreactors, and temporary immersion bioreactors. There are other types of bioreactors, such as diffusion bioreactors, magnetically stabilized fluidized bed bioreactors, immobilized plant cell reactors, disposable bioreactors, flow bioreactors, ultrasonic nutrient mist bioreactors, membrane bioreactors, etc., which differ in operation in a non-conventional manner.

In stirred tank bioreactors, agitation has been achieved by flat blade, marine, cell-lift, disk turbine impellers, magnetic stirrer, spindle with aeration tube, reciprocating plate, and rotating wall vessel to facilitate liquid circulation, mixing, and distribution of O_2 and nutrients. In order to reduce shear damage of the cells caused by mechanical agitation in stirred tank bioreactors, airlift and bubble column bioreactors are investigated for microprogation of several plant cells. However, cultures in these bioreactors may suffer from hyperhydricity and hamper the development. Temporary immersion of cells could be a possible solution and used to culture different plant species.

Because of the vast area of research, there are a large number of publications focusing on bioreactors for clonal propagation. Hence this paper only reviews the mechanical and biochemical features of different types of bioreactors using for plant biomass, metabolites, and enzymes production in micropropagation process and somatic embryogenesis, and their advantages and limitations for plant proliferation.

Key parameters for efficient bioreactor systems

A bioreactor system ensures the most optimal environment for growth and development of plant cells. However providing reuired nutrients and sufficient aeration to the culture without causing damage to the cells present a significant challenge for the design and operation of a well-functioning bioreactor. The key parameters associated with

bioreactor design include (i) shear–different systems may generate different amount of shear, which affect applicability of a system for shear sensitive or shear insensitive culture, (ii) gas exchange–ease of transfer of fresh oxygen in sterile condition into the bioreactor and removal of bi-product gases out of the system, and maintaining desired gas composition in bioreactor, (iii) sterility–ease of maintaining the bioreactor sterility throughout the entire culture process makes it a well-functioning system, (iv) exchange of medium–addition of fresh medium conveniently without disturbing sterility to restore nutrients level in the depleted medium or removal of spent medium improves performance of the bioreactor, (v) synchronization–a better performing bioreactor ensures the synchronized development of embryos. Details of these parameters and their influence on performance of bioreactors have been discussed below.

Shear– Shear stress is caused in both mechanically and pneumatically operated bioreactors due to mechanical agitation and aeration respectively. It plays a vital role in the suspension culture by affecting membrane integrity, cell growth, mitochondrial activity, size of clumps, morphology, release of proteins and phenolic compounds (intracellular metabolites), rate of formation of secondary metabolites, metabolic functions, enzyme levels in the cell, and even cell lysis (Choi et al., 1995; Namdev and Dunlop, 1995; Wongsamuth and Doran, 1997; Sun and Linden, 1999). At a favorable intensity, it affects cell growth and primary metabolism in plant cells by dispersing cell aggregates into suitable sizes, which favors nutrients and gas transfer and increases mitochondrial activity and intracellular protein content (protein expression) (Shi et al. 2003). However, plant cells are in general shear sensitive because they are relatively larger in size and have fragile, rigid wall and extensive vacuoles (Hooker et al., 1990; Scragg, 1995). Even a low shear stress (threshold varies from species to species) causes cell damage if it acts on the cells for a significant amount of time. Cell viability of *Perilla frutescens* reduced with shearing time in the rotating drum bioreactor in an experiment conducted by Zhong et al. (1994) in a low shear environment. They optimized an average shear rate of 20 to 30 s^{-1} caused by a marine impeller that resulted in maximum specific growth rate and yield.

Ballica and Ryu (1993) observed a significant drop in cell-yield at an aeration rate above 1.0 vvm in an internal loop airlift bioreactor of suspension culture of *Datura stramonium*. However the behavior of the culture in presence of stress varies with plant species. The cell growth of *Taxus cuspidata*, cultured in bubble column bioreactor, was seized in the first two to four days because of initial damage of cell membranes; however, by adapting the hydrodynamic shear environment through activating the defense genes (Shi et al., 2003) later on, cells had better growth and viability compared to the culture in shake flask (Zhong and Yuan, 2009). Production of some secondary metabolites, such as phytoalexin, lignin, etc., in a series of metabolic activities under stress environment

soothe the injury of plant cells while experiencing hydrodynamic stresses (Shi et al. 2003). Deposition of polysaccharide in the damaged area of the cell wall also facilitates the recovery (Sun and Linden, 1999).

The intensity of hydrodynamic shear stress can be measured in terms of shear force index and power input per unit mass; Kolmogoroff eddy length scale would be a measure of cell viability when the turbulent shear prevails in the bioreactor (Chen and Huang, 2000).

Gas exchange–Aeration plays a significant role for cell growth and production of secondary metabolites in bioreactors. For a well-functioning bioreactor, both the gas-liquid and liquid (medium)-solid (cells) mass transfers are important to conduct the respiration (metabolic process) of plant cells (Curtis, 2005). Carbon dioxide (CO_2), ethylene (C_2H_4), ethanol (C_2H_5OH), and acetaldehyde (CH_3CHO) are typically produced by respiring cultures (Thomas and Murashige, 1979; Perata and Alpi, 1991; Bieniek et al., 1995). Accumulation of these gases in the culture vessel inhibits growth of cultures (Jackson et al., 1985; Biddington and Robinson, 1991). Mostafa and Gu (2003) reported detrimental effect on cell growth and production of glycoprotein in a 1000 L stirred tank bioreactor due to increased level of dissolved CO_2. Schlatmann et al. (1993) observed that the gas composition was one of the reasons that affected the production of Ajmalicine in stirred tank bioreactor with forced aeration; excretion of ajmalicine to the medium was much less in a low ventilated system, such as a recirculation bioreactor. In a stirred tank bioreactor equipped with a dual impeller (flat-blade and pitched-blade), Huang et al. (2002) achieved a shake flask-comparable cell growth and L-DOPA production with controlled aeration (0.06 vvm) and agitation (at 300 rpm) of suspension culture of *Stizolobium hassjoo*. Their study showed high oxygen transfer rate played a vital role in higher cell growth and metabolite production. However they hypothesized that excessive aeration (0.1 vvm in their case) striped-off CO_2 and ethylene, considered as essential gaseous metabolites, and reduced the bioreactor performance. This can be manifested in the study on *Taxus chinensis* cell culture which revealed that both cell growth and production of taxuyunnanine C (TC) increased with increase in concentration of ethylene in bubble column bioreactor (Pan et al., 2000). However, Burg (1973) observed that ethylene inhibited cell division, and this might suppress embryo elongation (Bieniek et al., 1995).

Somatic embryogenesis of carrot is aided if the medium is saturated with dissolved oxygen (DO) (Jay et al., 1992; Teng et al., 1994). However, Kessell and Carr (1972) observed that the critical level of DO concentration for growth and embryo differentiation of carrot (*Daucus carota*) tissue in suspension culture was around 16% of saturation value above which carrot somatic embryogenesis was inhibited. The discrepancy may be due to the culture of different cultivars of carrot (Teng et al., 1994). Hvoslef-Eide et al. (2005) reviewed that Preil et al. (1988) and Preil (1991) showed the depletion of excess

CO_2 from the recirculating aeration system (silicone tube) helped to have improved growth of poinsettia cell cultures in bioreactors. In Applikon bioreactors for suspension culture of *Cyclamen persicum* Mill., Hohe et al. (1999) observed almost similar amount of CO_2 accumulation in the headspace (i.e. gas phase) and equal cell growth in bubble aeration system with headspace atmosphere in contact with outside environment through filter, and bubble free aeration system with recirculation of gas. As a control, in the Erlenmeyer flask they observed better gas exchange through the aluminum cap with eight times lesser CO_2 accumulation in the headspace, while having about four times cell growth compared to bioreactors. The cell growth increased with sweeping out CO_2 from the gas phase of bioreactor; on the other hand, more embryos developed in the bioreactor which accumulated CO_2. This was because of almost entirely presence of proembryogenic masses (PEMs) compared to that without CO_2 accumulation. In the latter case, large vacuolated cells were present along with PEMs.

Sterility–Plant cell culture can easily be contaminated by microbes since sucrose is a major ingredient of the culture media. So it has always been a logical concern to maintain sterilized environment inside the bioreactors during culture process which usually takes a long period of time (sometimes a few months). Especially liquid culture is more vulnerable to contamination than solid culture. Contamination of cell culture is not only associated with cost but also substantial losses of labor and time.

Exchange of medium– Ease of refreshing or changing the culture medium in the bioreactor, while maintaining the system sterility, is another requirement of a well-functioning bioreactor. In somatic embryogenesis, the developmental pathway of Norway spruce follows the sequential steps of initiation, proliferation, embryo differentiation, embryo maturation, desiccation, germination, and plant development (von Arnold et al., 2005). Each of these culture steps requires proper chemical treatment with step-specific medium. So exchange of medium is absolutely necessary for the continuation of culture process. The less complicated the exchange process is, the higher the probability of maintaining the system sterility.

Synchronization– Synchronization of somatic embryo development is considered as one of the most crucial issues for large-scale clonal propagation (Barry-Etienne et al., 2002) and still remains a bottleneck of implementing somatic embryogenesis in industrial scale (Molle and Fressinet, 1992). Asynchronized development along with abnormal morphology of somatic embryos, followed by maturation and germination, results in lower conversion to plants (Barry-Etienne et al., 2002). By synchronization, we here refer to simultaneous growth and development of somatic embryos while cultured in liquid and/or solid medium. Fully developed, polarized somatic embryos in the embryo differentiation phase can respond to maturation treatment and form mature somatic embryos (Sun, 2010). It may be assumed that

the cells in a cluster of PEMs receive different levels of nutrients due to their location within the cluster and this affects the growth of somatic embryos resulting in non-synchronous development. Low yield of plants from somatic embryos due to asynchronous development has hampered the practical implementations of somatic embryogenesis for large-scale clonal propagation of plants and agricultural goods. Bioreactors with the facility of disintegrating clusters to ensure nutrients at every location may be a good choice of achieving synchronous growth of somatic embryos.

Potentials and problems in bioreactor culture

Genetically identical plants, suitable for industrial scale operations, require a clonal propagation system that allows a scalable propagation in liquid medium. Using liquid medium reduces the manual operations and cost. It can easily be changed or refreshed during different phases of culture process so that the cultures can get the right nutrients at the right time. Because of this the liquid culture system can be adapted to automation. On the other hand, use of solid medium is labor intensive and costly.

However, liquid culture system is not free from problems. Hyperhydricity (Debergh et al., 1992) is a major bottleneck in this system. Stems and leaves are affected by hyperhydric malformations (Gaspar et al., 1995). Morphological abnorm-alities include shorter internodes in stems, and brittle, translucent, and wrinkled leaves (Debergh et al., 1981; Kevers et al., 1984; Gaspar et al., 1987; Paques and Boxus, 1987; Ziv 1991; Gaspar, 1991; Gaspar et al., 1995). Using temporary immersion bioreactors reduces the possibility of these malformations since the explants are not immersed in the nutrient medium constantly throughout the culture period.

Coagulation is another severe issue in liquid culture systems. This reduces the system's productivity and effi-ciency. In somatic embryogenesis, a large-scale clonal propagation of plants and agricultural goods is hampered due to asynchronous development of embryos resulting in low yields of plants. One explanation for this could be that the cells inside the somatic embryo clusters get lesser access to nutrients from culture medium in comparison to the cells at the surface of the PEMs cluster, resulting in different development of somatic embryos. It is important to keep immature embryos dispersed to maximize growth rate as well as probability of germination and successful plant formation; however embryos cannot grow in isolation. The cultured cells release conditioning factors to the surrounding medium. Egertsdotter et al. (1993) and Egertsdotter and von Arnold (1995, 1998) observed that not only the mechanical stresses from agitation but also the stress-related proteins released into Norway spruce (*Picea abies*) culture medium affect the development of somatic embryos from proliferation to maturation. Morphology of somatic embryos and number of produced suspension cells affect the release of conditioning factors.

Different designs of bioreactors

Mechanically operated bioreactors

Stirred tank bioreactor
A conventional stirred tank bioreactor consists of an impeller or agitator along with different ports for aeration, medium addition or removal, etc. Design of an agitator is becoming a real challenge to the researchers to maintain maximum cell viability. Stirred tank bioreactor has high volumetric mass transfer coefficients, and is enabled to maintain homogeneous nutrition environment in the culture medium through agitation (Huang and McDonald, 2009). However its high specific power input (P/V– power per unit volume), high energy dissipation rate, turbulence around the agitator, and shear damage of cells, tissues and embryos lead to consider other mechanisms of operating a bioreactor (Huang and McDonald, 2009).

Stirred tank bioreactors have been playing a significant role for several decades for clonal propagation. Such a bioreactor system was used by Jay et al. (1992) to study the effect of dissolved oxygen on carrot somatic embryogenesis (*Daucus carota* L.). Culture medium in a glass vessel bioreactor (3 L, working volume was 1.7 L) was stirred by an impeller consisting of four blades as shown in Fig. 1A. The process temperature was maintained 27°C with a water jacket. The bioreactor was connected with oxygen/nitrogen gas chamber, air compressor, and electronically controlled valve, and equipped with oxygen and pH probes. Dissolved oxygen concentration in the medium was maintained either at 100% (by supplying pure oxygen) or at 10% (by supplying pure nitrogen). Higher growth rate of cells during proliferation was observed in the culture medium having 100% concentration of dissolved oxygen because of low cytochrome oxydase activity (Atwell and Greenway, 1987; Jay et al., 1992). Similar trend was observed in somatic embryo production. This might be due to rapid synthesis of ethylene and acetaldehyde in hypoxic condition (Jackson et al., 1985; Small et al., 1989; Jay et al., 1992). Both ethylene (Tisserat and Murashige, 1977) and acetaldehyde (Perata and Alpi, 1991) showed inhibition characteristics of embryo production (embryogenesis) of carrot cells (Jay et al. 1992). Similar stirred tank bioreactor system was used by Jay et al. (1994) to investigate the effect of pH of culture medium on carrot cells' growth. HCl (1N) and KCl (1N) were used to maintain the media pH of 4.3 and KOH (1N) was used for pH of 5.8. In absence of 2,4-dichlorophenoxyacetic acid (2,4-D), more embryos were produced in the media of pH of 4.3 compared to that of pH of 5.8. However plantlet formation occurred more in the later. This may be associated with lower uptake

rate of ammonium (NH_4^+) and sugar by the embryos in the more acidic medium (pH = 4.3). Jay et al. (1994) noted that the inability to release protons by the embryos limited the nutrients uptake, as observed by Schubert et al. (1990) in case of root culture of field beans (*Vicia faba*).

Depending on requirement and applications, researchers have developed several different designs of stirred tank bioreactors. A few examples are shown in Fig. 1.

Rotating drum bioreactor

Rotating drum bioreactor is being used in plant cell culture as an alternate of stirred tank bioreactor to achieve higher mass transfer and cell viability. In a comparative study between stirred tank and rotating drum bioreactors, Tanaka et al. (1983) observed a lower cell growth of *Vincarosea* in stirred

tank bioreactor, equipped with baffles, air sparger, and flat six-blade impeller, agitated at a rpm of lower than 115 or higher than 170. This could possibly be due to insufficient transfer of O_2 to the cells at a lower rpm, or damage of the cells because of hydrodynamic shear at a higher rpm. The study showed that rotating drum bioreactor performed more efficiently to supply oxygen to the cultures than stirred tank bioreactor under highly viscous and low hydrodynamic shear conditions. The drum, made of polycarbonate, consisted of air supply and vent ports and an oxygen sensor as shown in Fig. 2. The experiment was conducted in three sizes of drum, 1 L, 2.4 L, and 4 L. The two types of bioreactors were compared under a constant air flow rate of 1 vvm to maintain the same $k_L a$ value (volumetric oxygen transfer coefficient) in both cases.
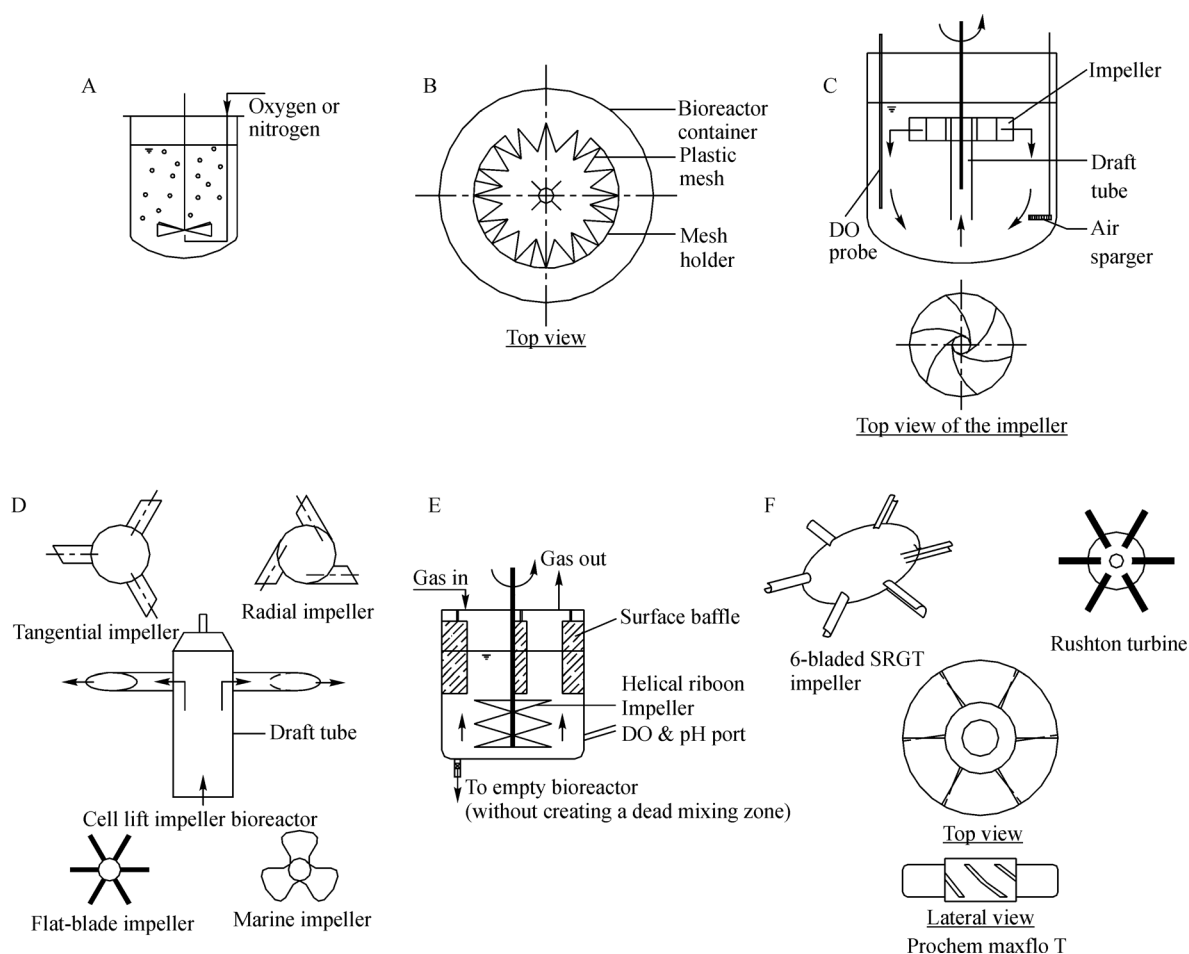

Figure 1 Different designs of stirred tank bioreactor; rotation of impeller in the bioreactor provides agitation to the liquid medium that helps to maintain homogeneity of nutrient and O_2 in the medium. (A) Schematic of a conventional stirred tank bioreactor (Jay et al.,1992). (B) Stirred tank bioreactor with plastic mesh for immobilization of hairy roots (Cardillo et al., 2010). (C) Centrifugal impeller stirred tank bioreactor that provides an axial inlet and radial outlet of medium causing better mixing, low shear, and higher liquid lift (Wang and Zhong, 1996). (D) Cell lift impeller bioreactor in which cells along with medium are lifted through the draft tube of the impeller and result In either tangential or radial exit of cells, conventional flat bed and marine impellers were also used for comparison (Treat et al., 1989). (E) Bioreactor with double helical ribbon impeller that pumps liquid upward when rotates counter clockwise (Jolicoeur et al., 1992). (F) Rushton turbine and Scaba (SRGT) impellers (Amanullah et al., 1998); in Scaba impeller six curved parabolid blades were used and its rotation resulted in radial flow of fluid in the bioreactor.
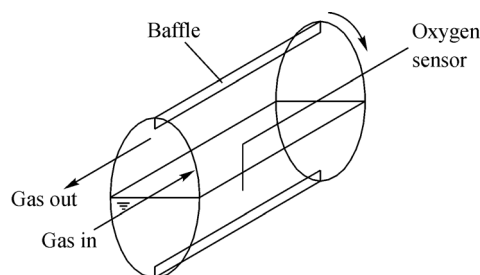
Figure 2 Rotating Drum Bioreactor constructed with a vessel having baffles inside contains the culture and the medium at the bottom. Rotation of the vessel facilitates mass transfer under low shear condition (Tanaka et al.,1983).

Fung and Metchell (1995) studied the performance of baffles in rotating drum bioreactor while performing SSF (solid-state fermentation: fermentation on moist solid substrate in absence of or near absence of free liquid (Pandey, 1992; Domínguez et al., 2001)) of *Rhizopus oligosporus*. They observed about 58% rise of oxygen uptake rate in a baffled reactor over a non-baffled one based on the weight of initial dry substrate. A stainless steel drum of 10.9 m long and 0.56 m in diameter had four evenly spaced baffles of width 0.17 m (ratio of baffle width to drum diameter was 1:3.29) were placed along the length of the drum. Air entered and left at two ends of the drum at a rate of 24 L/min. During operation, they noticed a higher temperature of the culture in the baffled bioreactor that might possibly be due to rapid metabolic heat generation because of better aeration in the system and concluded that number and shape of the baffles, along with rotational speed, and aeration rate might play a significant role to optimize culture process in rotating drum bioreactor.

Pneumatically operated bioreactors

Bubble column bioreactor
Bubble column is one of the early inventions among pneumatically operated bioreactors. A conventional bubble column bioreactor, shown in Fig. 3A, consists of a cylindrical vessel having an air sparger at the bottom. Because of its high heat and mass transfer rates, plug free operations, and low operating and maintenance cost (Kantarci et al., 2005), bubble column bioreactor has been playing an important role in large scale production of high value plants and metabolites.

Arcuri et al. (1983) produced thienamycin using a continuously operated bubble column bioreactor as shown in Fig. 3B. *Streptomyces cattleya* cells were immobilized by attaching them with celite particles. A cylinder, referred to as a solid-liquid disengagement cylinder, was placed inside the reactor to separate celite-cell aggregates from the medium during continuous operation.

Instead of using a vertical side wall bubble column reactor, Fujimura et al. (1984) studied continuous production of L-

arginine from *Serratia marcescens* cells in a cone shaped bioreactor (jacketed), shown in Fig. 3C, using immobilized cell culture procedure. The shape of the bioreactor could possibly eliminate the dead zone and provide better mixing of nutrients and mass transfer to the cells. About 3×10^{10} cells were entrapped per ml of carrageenan gel beads. Oxygen played a key role for the growth of *S. marcescens* cells. Concentration of L-arginine in the medium increased with increase in oxygen concentration. Since the gel behaved as a diffusion-barrier, higher concentration of dissolved oxygen in the medium provided sufficient oxygen diffusion for growth of the cells. El-Sayed and Rehm (1987) investigated the performance of a conventional bubble column bioreactor having a straight vertical vessel and a conical shaped bubble bioreactor through continuous production of penicillin by *Penicillium chrysogenum* cells entrapped in Ca-alginate beads for immobilization. They observed that at a certain air flow rate, conventional bubble column bioreactor lost fluidization partially because of swelling of the beads due to cell growth. However it was not an issue for the conical bubble reactor; instead, lower mechanical abrasion at the wall among rotating beads and better distribution of nutrients and oxygen were achieved.

A bubble column bioreactor with cylindrical loofa sponges was used by Ogbonna et al. (2001), shown in Fig. 3D, to produce ethanol from sugar beet juice (as a substrate) by *Saccharomyces cerevisiae*. The function of loofa sponges was to immobilize the cells. They proposed external loop (bubble column) bioreactor which could immobilize the cells uniformly on loofa sponges at different sections and produce ethanol efficiently in a large scale.

Use of bubble column bioreactor for large scale clonal propagation of plant was investigated by Akita et al. (1994) using a 500 L bioreactor. The shoots of *Stevia rebaudiana* were used in this experiment. A porous disc of 300 mm diameter was attached at the bottom of the bioreactor as a gas sparger. There were four fluorescent lamps, with an approximate intensity of 1000 lx. each, inside the bioreactor. Inoculum size was 460 g (Fresh weight) in 300 L of sterilized culture medium. Compressed air was sparged into the medium at a rate of 15 L/min. Shoots grew very well having a final weight of approximately 64.6 kg (Fresh weight), though damage occurred by air bubbles just above the gas sparger, and gradual decrease in growth was observed away from fluorescent lamps.

Airlift bioreactor
Airlift bioreactor is introduced for plant cell culture to provide a low hydrodynamic shear environment. It reduces the loss of cell viability compared to stirred tank bioreactor and eliminates the dead zones that cause inefficient mixing in the bubble column bioreactor. An airlift bioreactor is operated by sparging gas (or air) in liquid medium placed underneath a draft tube of the bioreactor. This causes a pressure difference

between inside and outside of the draft tube and results in a fluid circulation in the bioreactor.

A conventional airlift bioreactor consists of a sparger, a bioreactor vessel, a solid vertical side draft tube, known as riser and placed inside the bioreactor vessel, and several ports as needed. Researchers have made modifications of this initial design in order to improve the bioreactor performance, such as mixing, shear rate, growth rate of cells, biomass yield, and oxygen transfer rate (Smart and Fowler, 1984; Fu et al., 2003; Chen et al., 2010). Smart and Fowler (1984) investigated the efficiency of a simple air lift bioreactor in terms of aeration rate, mixing, growth rate of cells, and biomass yield in the suspension culture of *Catharanthus roseus*. The bioreactor consisted of a vessel (5 l LKB Ultraferm fermenter) and a 94 mm diameter draft tube placed above an air sparger ring, shown in Fig. 4A, having 17 holes of 0.533 mm in diameter each. The relation between aeration and agitation was established in terms of superficial gas velocity (V_s) and mixing time (t_m) as follows,

$$t_m - 0.032 \frac{H}{\sqrt{V_s}} \left(\frac{D}{d} \right)^2 \qquad (1)$$

where, H and D are the height and diameter of a fermenter respectively, and d is the diameter of the draft tube. The system geometry and ratios of H/D and d/D played a significant role in mixing. Growth rate of plant cells cultured in this experiment reduced with aeration over a certain rate. Design of the draft tube was modified in an experiment performed by Townsley et al. (1983) where they used a conical shaped draft tube above the air sparger ring in a larger vessel, shown in Fig. 4B, for the suspension culture of selected *Tripterygium wilfordii* for the production of tripdiolide. The cone shaped draft tube was used to provide sufficient circulation at the bottom of the vessel. The plant cells were pushed up by air through the draft tube, and with decrease in cross-sectional area of the draft tube along the flow path, the velocity of cells and medium increased. Hence the cells started falling at a higher velocity outside the draft tube. This way an efficient mixing was achieved during the culture process. A small magnetic stirrer was used in this system along with the draft tube. Air was recycled in this experiment by controlling the level of oxygen in the air. Increased biomass yield as well as tripdiolide production were observed.

A comparative study of bioreactor performance among stirred tank, bubble column, conventional airlift bioreactors and a bioreactor with mesh/net draft tube, shown in Fig. 4C, was carried out by Chen et al. (2010) for the production of
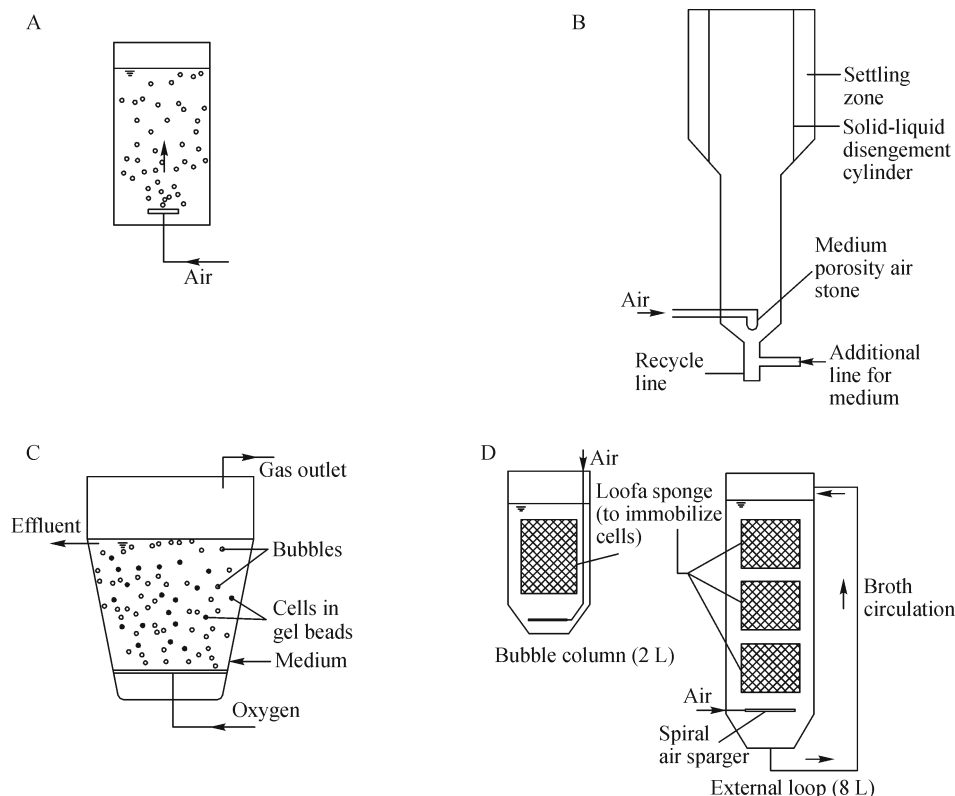

Figure 3 Bubble column bioreactor. (A) Schematic of a conventional bubble column bioreactor having an air sparger at the bottom that generates air bubbles and provides agitation in the medium. (B) Arcuri et al. (1983) has used a settling zone for cell aggregates in the bubble column bioreactor for continuous operation (bubble formation and medium perfusion). (C) Cone shaped reactor working similar to conventional bubble column bioreactor reduces the possibility of dead zones (Fujimura et al., 1984), (D) bioreactor with cells immobilized on loofa sponges sparging air from the bottom and having medium circulation through an external loop (Ogbonna et al., 2001).

chitinolytic enzymes from suspension culture of *Paenibacillustaichungensis*. Under same operating conditions of the volume of culture medium, aeration rate, and inoculated culture, the net draft tube bioreactor showed a little improvement in chitinolytic enzyme yield (~2.94%) and productivity (~4.26%) over the conventional airlift bioreactor. The enzyme activity and the cultivation time were same in both cases. However this newly designed bioreactor showed much better performance over bubble column reactor, but the stirred tank bioreactor had same productivity as that of the net draft tube airlift bioreactor.

To investigate the improvement of productivity, Sajc et al. (1995) developed an external loop airlift bioreactor for production and continuous extraction of extracellular metabolities (anthraquinones) by immobilized culture of *Frangula alnus* Mill. plant cells (Fig. 4D). The results showed much higher production of anthraquinones compared to shake flask culture of immobilized cells. Solvents, such as silicone oil and n-hexadecane, were used in different runs to facilitate extraction of metabolites. Frequent replacement of the solvent with the fresh one showed higher yields of anthraquinones, because with time the solvent became saturated with the products.

Temporary immersion bioreactor
The advantage of temporary immersion over suspension culture is that the explants in the temporary immersion bioreactor are not immersed permanently in liquid medium and, therefore, eliminates hyperhydricity that may be caused

by excessive accumulation of water in the tissues. Hyperhydricity may cause cell damage due to depletion of O_2, formation of reactive oxygen species (ROS), and oxidative stress induction in the cells (Ziv, 2005). All temporary immersion systems so far been developed have been working on similar technology, i.e., the explants are kept away from the medium, and the medium is stored in a separate (usually lower) compartment in the same vessel where the explants are placed, or in a separate reservoir(s). The medium is then pushed by air to immerse the explants to provide nutrients and at the same time to refresh the gas composition in the headspace. After a period of immersion, the liquid medium returns to the reservoir either by gravity or suction. This process may take place once or more in 24 h period with an air pump, solenoid valve, timer, etc.

An automated temporary immersion culture system, shown in Fig. 5A, of plant propagules was developed and tested with orchid tips, aster shoot tips, cow tree shoot tips, date palm, and carrot callus by Tisserat and Vandercook (1985). An elevated (30 to 45 cm above the plane on which the rest of the system was placed) polystyrene container of 320 mm long, 175 mm wide, and 165 mm high was used as a culture chamber. Sometimes a plastic tray with 10 to 28 wells was used inside the chamber for the purpose of containing the plantlets or callus on it. The culture chamber was connected through silicone tubings with pumps, 3-way valve (stainless steel), and reservoirs of culture media. Air filters were used to avoid contamination. The system was operated with computer and electronic circuitry to ensure an automated consistent
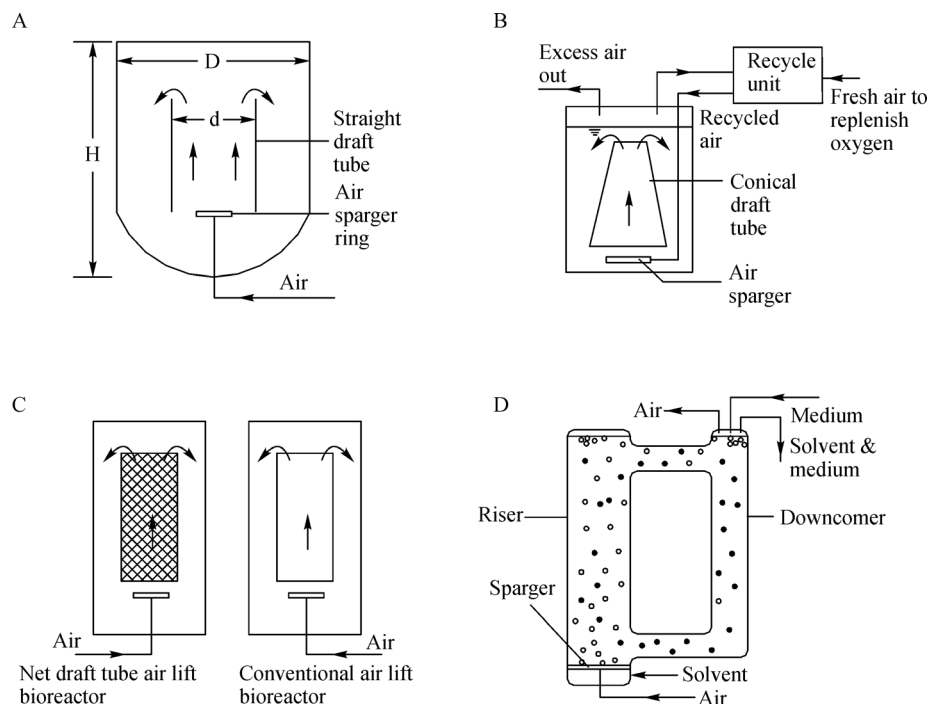

Figure 4   Various designs of airlift bioreactors in which the air or gas is sparged underneath the draft tube of the bioreactor; this causes a pressure difference between inside and outside of the draft tube and results in a fluid circulation in the bioreactor. A–C: Internal loop. D: external loop. (A) Smart and Fowler (1984), (B) Townsley et al. (1983), (C) Chen et al. (2010), and (D) Sajc et al. (1995).
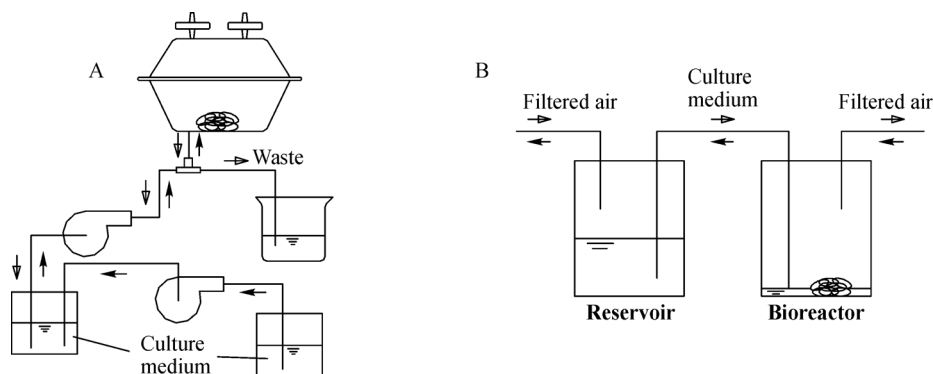
Figure 5   Temporary immersion bioreactor in which the culture and the medium remain in separate containers or in different compartments in the same container so that the culture does not immerse in the medium continuously; instead the medium is pumped to the culture container/compartment, culture is soaked with the medium, and then the medium is drained out. (A) Tisserat and Vandercook (1985) and (B) Escalona et al. (1999).

supply of nutrients periodically to the explants during the culture process. The culture medium was replenished when necessary.

Alvard et al. (1993) investigated six different culture conditions for banana meristem.The explants were cultured on semi-solid medium, and in liquid media with complete immersion, partial immersion, temporary immersion (20 min in 2 h), continuous air bubbling at 20 L/h, and cellulose culture support having the bases of explants in contact with the medium. The most promising outcome came from the temporary immersion technique in which multiplication rate was the highest without hyperhydricity. Shoots used as initial material for this study were obtained from successive proliferation subcultures on gelled medium. The bioreactor used in the temporary immersion culture system consisted of two compartments. Explants were placed in the upper compartment and the lower compartment was filled with culture medium. The upper compartment was connected with an air filter; the lower compartment was connected to an air pump, filter, and 3-way solenoid valve. During operation, the lower compartment was pressurized with air, which pushed the culture medium to the upper compartment through a connecting tube. The explants were immersed in the medium for a while (20 min); air bubbles agitated the cultures during immersion, and refreshed the area in the bioreactor head space by scavenging the exhausted air. The pressure in the lower compartment was released with the help of the solenoid valve to bring the medium back to the lower compartment when the immersion process was over. This process was repeated every 2 h. The culture medium might be refreshed, or changed manually for stimulating different phases of tissue growth.

Escalona et al. (1999) performed a comparative study of pineapple micropropagation using solid medium, conventional liquid medium technology, and temporary immersion bioreactor. The pneumatically driven temporary immersion system, shown in Fig. 5B, consisted of two containers, one as a medium reservoir and the other one as a culture vessel, connected with glass and silicone tubes, filters, solenoid

valves and air pumps. The liquid medium was pushed by filtered air to the container containing the explants (shoots of 2 to 3 cm in length) every 3 h using an air pump. The medium kept the explants fully immersed for 2 min and was then pushed back by filtered air in the reverse direction. Filter paper was used to avoid continuous contact of liquid medium with the explants. It was possible to have a saturated filter paper with spent medium because of high frequency of operations (8 times in 24 h period); hence the spent medium might come in contact with new medium during the successive phases of cell culture. Also, because the high pressure air pushed the medium out of the culture vessel, this (pressure) would develop higher normal stresses on the propagules. The effect is not known yet. Similar design of temporary immersion system was used by Lorenzo et al. (1998) for sugarcane shoot formation and Jiménez et al. (1999) for production of potato microtubers.

Diffusion bioreactor

Suspension cell culture of *Thalictrum rugosum* was carried out in a reactor equipped with hydrophobic polypropylene membrane, either arranged as a coil or a basket (Piehl et al., 1988). The purpose of the membrane was to supply oxygen to the medium through diffusion. The rotation of membrane coil or basket by means of magnetic stirrer or eccentric shaft provided a gentle agitation to the medium as well as the cultures. This reduced the hydrodynamic shear stress, which would otherwise be caused by gas sparging or agitation mechanisms, and damage the tissues. No foam formation by damaged cells or cell debris was observed. Cell adhesion might cause clogging of the membrane. However this was not observed during the culture process. Instead of using a polypropylene membrane, Luttman et al. (1994) used silicone tubing to diffuse oxygen into the medium of a bioreactor, shown in Fig. 6, for suspension culture of *Euphorbia pulcherrima,* and *Clematis tangutica*. The silicone tube, working as a permeable membrane for oxygen, nitrogen, and
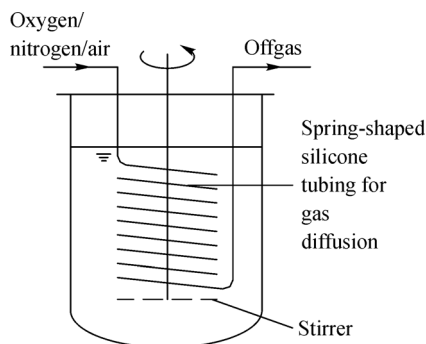
Figure 6 Diffusion bioreactor having a helical shaped silicone tube permeable to O_2, N_2, and air that diffuse to the medium from the tube and the stirrer agitates the medium containing the culture (Luttman et al., 1994).

air, was given a helical shape with the help of a steel helical spring and inserted into the bioreactor. The dimension (diameter, length, and wall thickness) of the tube was decided based on the amount of embryogenic biomass to be produced. The chance of foam formation was much lower in this bioreactor because no bubble was produced during operation (unless the aeration pressure was raised above the bubble point).

Perfusion bioreactor

Perfusion bioreactors have been playing a vital role in the production of secondary metabolites. They are involved in continuous feeding of nutrients and harvesting of metabolites from spent medium during the culture period (Langer, 2011). They may be operated either mechanically (Su and Arias, 2003; De Dobbeleer et al., 2006) or pneumatically (Su et al., 1996). Several approaches (De Dobbeleer et al., 2006) have been applied in these bioreactor systems for an efficient removal of bio-products from the medium with a reduced degradation of metabolites. For example, continuous extraction of alkaloids secreted during hairy root culture was possible by circulating silicon oil in a two-liquid-phase bioreactor (Tikhomiroff et al., 2002); increased productivity of secondary metabolites was achieved by using resins in situ for cell suspension systems (Williams et al., 1992; Lee-Parsons and Shuler, 2002). Harvesting and purification of secondary metabolites were improved by using of resin (XAD-7) externally in the medium circulation loop (Klvana et al., 2005).

However, the major constraint of recovering metabolites from the culture medium was to retain majority of the cells by keeping the cells and the medium separate during perfusion culture. In achieving this, three techniques were widely used-centrifugation, filtration, and sedimentation (Su et al., 1996; Seki et al., 1997).

Filtration might have clogging problem (Tokashiki et al., 1990), and centrifugation might damage plant cells because of their shear sensitivity (De Dobbeleer et al., 2006). So

gravitational sedimentation was considered as the most efficient way to retain the cells and separate the medium containing secreted metabolites (Wang et al. 2010). However this method required an optimization of perfusion rate of the fresh medium, recirculation rate of the culture medium, and cell retention.

Attree et al. (1994) proposed a low-cost method based on perfusion technology to generate mature white spruce somatic embryos. A flat absorbent pad, used as a culture pad to support the embryos, was placed above the liquid culture medium in the cuboid shaped polypropylene culture chamber. This pad consisted of cotton wool, 48 μm nylon mesh and a filter paper. The bioreactor was connected with the culture medium and spent medium reservoirs at two diagonally opposite corners of the base. The culture medium was pumped into the culture chamber at a rate of 60 mL/d for 7 weeks. The excess medium was collected in the spent medium reservoir by gravity. High quality matured embryos were recovered in this process. These embryos had higher desiccation tolerance and postgerminative growth compared to those cultured on agar medium.

Gravitational sedimentation technique was used by Su et al. (1996) for the suspension culture of *Anchusa officinalis* and continuous production of secreted protein throughout the culture period in a perfusion external loop airlift bioreactor, shown in Fig. 7A. A baffle was placed vertically to have a settling zone in the upper portion of the downcomer. Continuous removal of the medium with secreted metabolites was taken place from the settling zone and the perfusion medium was fed from the bottom of the riser to replenish nutrients for the cells. They achieved amaximum cell density of 2.16 times and an extracellular protein concentration of 2.36 times more in their system compared to batch culture.

A stirred tank perfusion bioreactor of 3.3 l working volume was used for the production of acid phosphatase (APase) while culturing *A. officinalis* (Su and Arias, 2003). The bioreactor had an annular settling zone for continuous separation of cells and medium. The schematic of the bioreactor is shown in Fig. 7B. The bioreactor was made of glass with an annular stagnant zone because of the presence of a cylindrical baffle. An agitator with six blades on top (Rushton turbine) and three blades (to pump in upward direction) at the bottom was used for mixing. A sintered glass tube gas sparger of 140 μm pore size was placed below the impeller. Complete cell retention was possible at a perfusion rate of up to 0.4 vvd (vessel volume per day) achieving the APase production of about 300 units/(L·d) with a cell dry weight exceeding 20 g/L. The culture operation at a high packed cell volume (PCV over 70%) was avoided owing to declined oxygen uptake and reduced cell viability. Cells were removed from the bioreactor via bleed stream (at a rate of up to 0.11 vvd) that led to higher APase production and increased cell dry weight.

De Dobbeleer et al. (2006) developed a perfusion bioreactor, consisting of four sedimentation columns, a gas

sparger, and a double-helical ribbon agitator (shown in Fig. 7C) to extract secondary metabolites continuously from the cell suspension culture of *Eschscholzia californica*. The rotational speed of the agitator was optimized with the gap between the bottom of the sedimentation column and the top of the helical ribbon to have a stable cell sedimentation front and to minimize cell loss. The liquid medium with secreted secondary metabolites was circulated between sedimentation columns, fluidized resin (XAD-7 placed in an extraction column in the outer loop), and the bioreactor by a peristaltic pump.

Stirred tank perfusion bioreactor with a separate sedimentation unit was proposed by Wang et al. (2010). The bioreactor unit was equipped with a 4-blade agitator, gas sparger, and pH and dissolved oxygen probes. The spent medium was continuously removed from the bioreactor to the external sedimentation unit via overflow, and perfusion medium was fed into the bioreactor to replenish nutrients. As cell density increased in the bioreactor, more cells entered the settling column. So a harvest pipe was attached underneath the sedimentation unit to have cell bleed which led to high cell growth and reduced dead cell accumulation.

A few special types of bioreactors

Magnetically stabilized fluidized bed bioreactor
The concept of having fluidization of magnetized particles by a stream of fluid in presence of a uniform magnetic field (Rosensweig, 1979) was applied to design a magnetically stabilized fluidized bed bioreactor (Bramble et al., 1990). This

bioreactor avoided the hydrodynamic instability and, therefore, achieved turbulent free, plug flow of solids. A schematic of this bioreactor is shown in Fig. 8A. The cells were attached to gel beads containing magnetizable solids. Then they were added to the bioreactor from the top and moved downward in a "lockstep fashion." After a certain period of culture, beads along with the spent cells were removed from the bottom using oxygen-enriched recycled and fresh liquid media that made the bed fluidized. Major advantages of this bioreactor includes high rate of oxygen transfer while causing a low-shear to the cells and elimination of support damage which would otherwise have resulted from particle collisions.

Immobilized plant cell bioreactor
In a plant cell bioreactor, the cells were immobilized by filtering them on a cotton matrix (commercially available terry cotton sheet) (Choi et al., 1995). About 85–90 g fresh weight of *Gossypium arboreum* cells were entrapped on a cotton sheet of 400 cm^2. To strengthen the immobilization of cells, the cotton cloth was spirally wound with a spacer (Goodloe 304 stainless steel woven packing). This way shear force on the cells by the immobilization matrix was eliminated. The immobilized cells were then placed in a plug flow type reactor (300 mL) as shown in Fig. 8B.

Reciprocating plate bioreactor
A reciprocating plate bioreactor, named after reciprocating motion of a plate stake in a bioreactor as shown in the schematic (Fig. 8C), was used to investigate its potential for cell suspension culture. *Vitis vinifera* cells were used as a
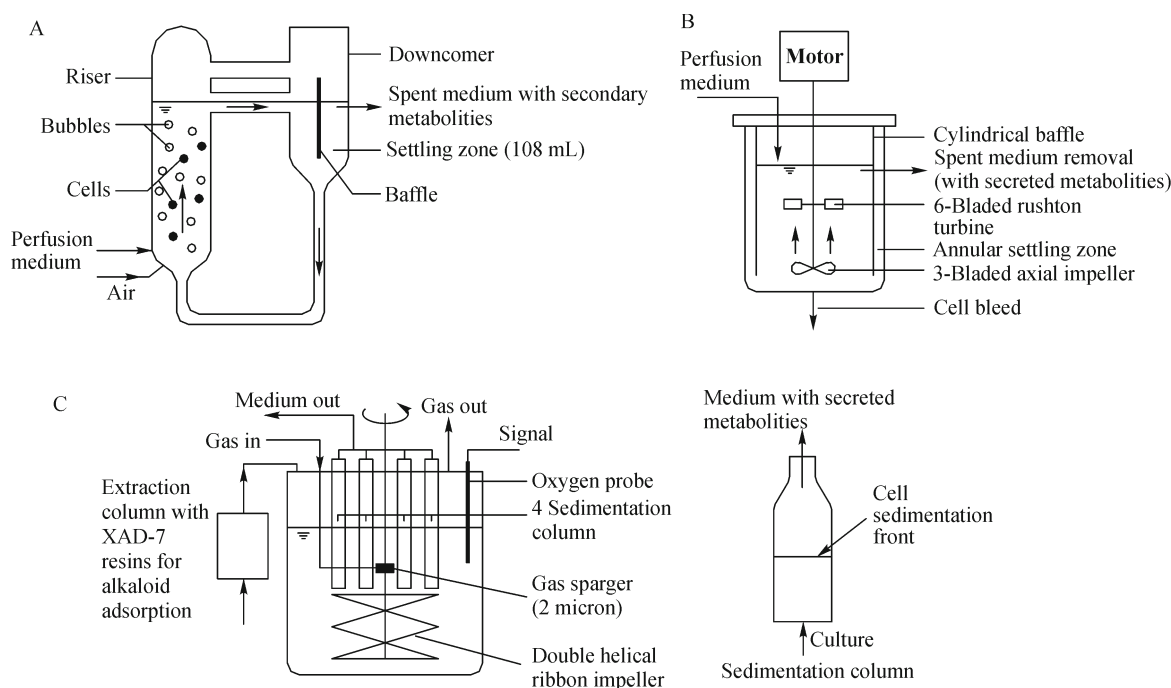

Figure 7   Perfusion bioreactor. (A) Su et al. (1996), (B) Su and Arias (2003), and (C) De Dobbeleer et al. (2006).

model culture in this bioreactor for their growth and viability (Gagnon et al., 1999). The bioreactor vessel was built with two concentric stainless steel tubes providing 17 L of working volume. The bioreactor consisted of six perforated stainless steel plates attached equispaced to a shaft operated by a variable speed motor. This resulted in a reciprocating motion to the plates and provided agitation and mixing in the culture medium. A gas sparger was placed at the bottom to provide aeration (or nitrogen) to the culture. The authors observed a significant decrease in O_2 transfer coefficient with increase in cell concentration in the bioreactor.

Flow bioreactor

Kino-Oka et al. (1999) explained two types of flow bioreactors, namely single-column reactor (1 L) and radial flow reactor (1.5 l), used for high density hairy root culture of red beat. In single-column reactor, medium flowed from the top to the bottom of the reactor through hairy root cultures anchored to a stainless steel mesh (Fig. 8(D-i)). In radial flow reactor (Fig. 8(D-ii)) the hairy roots were anchored to stainless steel wires in between two concentric stainless steel meshes and fresh medium was introduced radially through four ports at the outer vessel. The spent medium was discharged from the two ports at the center of the reactor. Each reactor was connected to a medium aeration unit and a humidifier for operation.

Disposable bioreactor

Disposable bioreactor, with its name implying a one-time use culture vessel, was introduced to reduce production costs (Terrier et al., 2007), cross-contamination (Eibl et al., 2010), and cell damage due to gas bubbles and mechanical agitators (Singh, 1999). A disposable bioreactor causing wave agitation to the medium by rocking motion is shown in Fig. 8(E-i) (Singh, 1999). The container of a disposable bioreactor is usually made of polyethylene, polystyrene, polytetrafluoroethylene, polypropylene, or ethylene vinyl acetate (Eibl et al., 2010). The rocking motion of the wave bioreactor was replaced by the wave and undertow (WU) mechanism and demonstrated by Terrier et al. (2007), shown in Fig. 8(E-ii), for the production of isoflavones from tobacco and soya cell cultures. A 100 L working volume WU bioreactor container was placed on a horizontal table which was equipped with a platform at the side that rose periodically to induce wave and undertow, and resulted in agitation and aeration in the culture medium. To investigate the performance of a pneumatically operated disposable bioreactor, the authors designed a 70 L working volume bubble column bioreactor with a vertical, flexible plastic cylinder, named slug bubble (SB) bioreactor that intermittently generated a large bubble occupying almost the entire cross-section of the tube (Fig. 8(E-iii)). When the bubble moved up, a thin film of medium flowed downward along the wall as a falling film and an enhanced mixing and mass transfer occurred at the rear of the bubble.

Membrane bioreactor

Membrane bioreactor provides a convenient means of aeration to the cell culture in bioreactor without generating bubbles, and therefore, reduces the possibility of cell damage due to hydrodynamic shear. It may also be featured with a special membrane for specific molecular cut-off (MWCO) to supply nutrients to the cells and for separation of metabolites. This feature was introduced in a two-compartment (nutrient medium compartment and cell compartment) bioreactor (Fig. 8(F)), named CELLine 350 from Integra Biosciences, Chur, Switzerland. It was used for the production of Human α-1 antitrypsin protein from transgenic rice cell culture (McDonald et al., 2005). About 7–10 mL of concentrated rice cells were packed in the cell compartment which was in between a 10 kDa MWCO membrane and a gas exchange membrane. Initially the medium compartment was filled with 65 mL of medium in addition to 25 mL which was poured in to wet the MWCO membrane. For homogeneity of nutrients in the medium, the bioreactor was agitated at 70 rpm using an orbital shaker in the dark at 25°C. Instead of a separate gas exchange membrane, the aerated medium was fed into a tubular membrane bioreactor to enhance the extraction of secondary metabolites from *Beta vulgaris* and *Catharanthus roseus* cells using a low-level electric current (Yang et al., 2003). A ceramic membrane tube, made of high purity alumina with hydrophilic property of the surface, was used to separate the cells from the flow of medium in the bioreactor. The membrane bioreactor is suitable for reduced shear on cell walls, improved supply of nutrients and O_2 to the cells, and ease of separation of cell-secreted products since these are retained in the cell compartment. However due to expansion of cell compartment because of osmotic flux and increased biomass (McDonald et al., 2005), low durability of the MWCO membrane might restrict long-term and scaled up cell culture in membrane bioreactor.

Conclusions

We have reviewed different designs of bioreactors used in plant cell culture for plant propagation and production of secondary metabolites. Design of a well-functioning bioreactor depends on the liquid circulation, mixing, and aeration for distribution of oxygen and nutrients for optimal growth of plant cells. The bioreactor design is technically challenged by the properties of the culture, such as viscosity, shear sensitivity of cells, tendency of cell aggregation, and foam formation. Especially the shear sensitivity of cells plays a significant role, because the plant cells have a low shear tolerance. This led to several novel designs of bioreactors which have become successful in production of clonal propagation of plants and secondary metabolites; some of them have also been commercialized. Mechanically operated bioreactors have been serving the arena of plant cell and tissue culture for quite a long time due to its high volumetric
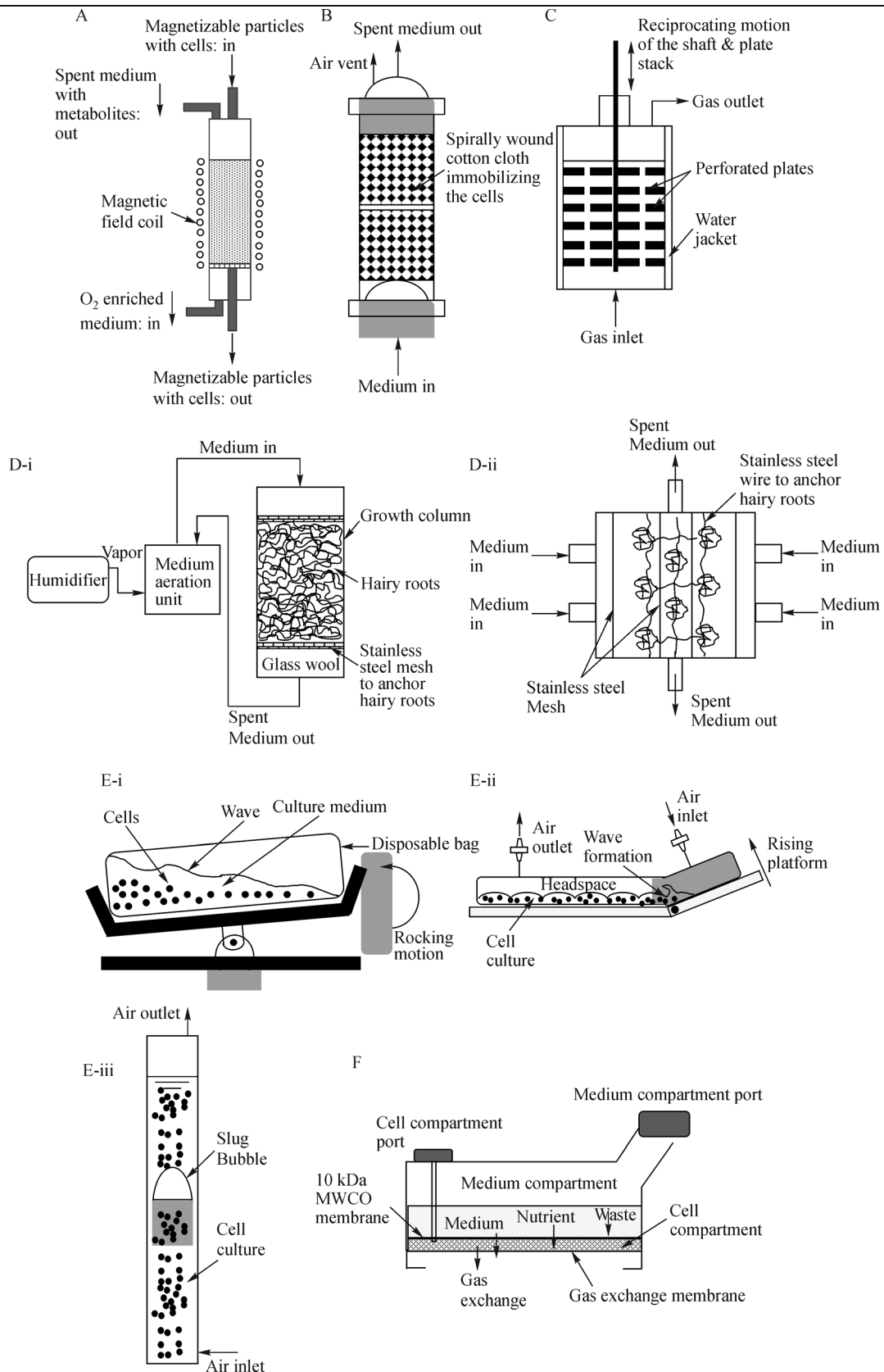
Figure 8 A few special types of bioreactors. (A) Magnetically stabilized fluidized bed bioreactor (Bramble et al., 1990). (B) Immobilized plant cell bioreactor (Choi et al., 1995). (C) Reciprocating plate bioreactor (Gagnon et al., 1999). (D) Flow bioreactors– (i) Single-column reactor, (ii) Radial flow reactor (Kino-oka et al., 1999). (E) Disposable bioreactors– (i) Wave bioreactor (Singh, 1999), (ii) Wave and Undertow bioreactor (Terrier et al., 2007), (iii) Slug bubble bioreactor (Terrier et al., 2007). (F) Membrane bioreactor (McDonald et al., 2005).

mass transfer coefficients and inhibiting cell coagulations; however, its application is limited because it causes high shear stress and thereby reduces cell viability. On the other hand, pneumatically operated bioreactors have low shear effect on cells and tissues. In addition to that, temporary immersion bioreactors have not only eliminated the high shear on the cells but also reduced the chance of morphological abnormalities of tissues due to hyperhydricity. Membrane bioreactor, magnetically stabilized fluidized bed bioreactor, and immobilized plant cell bioreactor have also eliminated the adversity of hydrodynamic shear on cells due to agitation and aeration.

Compliance with ethics guidelines

Nazmul H.A. Mamun, Ulrika Egertsdotter, and Cyrus K. Aidun declare that they have no conflict of interest.

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