Bioreactor Technology for Hairy Roots Cultivation

17

Shakti Mehrotra, Sonal Mishra, and Vikas Srivastava

Contents

S. Mehrotra (✉)
Plant Biotechnology Division, Central Institute of Medicinal and Aromatic Plants, Lucknow, India
e-mail: shaktimehrotra22@gmail.com

S. Mishra
National Institute of Plant Genome Research, New Delhi, India
e-mail: mishrasonal.nipgr@gmail.com

V. Srivastava
Department of Plant Sciences, Central University of Jammu, Jammu (J & K), India
e-mail: vikassrivastava25@gmail.com; vikassrivastava@cujammu.ac.in

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Abstract

Bioreactor technology is an integral requisite to the development of scale-up production process of many plant-based high-value products. The proper selection and design of the bioreactor is required to determine the optimal industrial scale bioprocess and the subsequent capital investment. A primary cause of the lack of success in commercial production of secondary compounds using hairy root culture systems is their low yield. To increase the production of hairy root-based bioactive compounds, several strategies, like elicitation, metabolic engineering, and up-scaling, etc., have been adopted. Out of these strategies, the up-scaling in bioreactor deals with the principle of large-scale metabolite production in proportion of high biomass growth. The goal of an effective bioreactor is to control, contain, and positively influence a biological reaction in an incessant way in order to get desired productivity. This chapter provides a descriptive account on up-scaling of hairy root cultures for various purposes including secondary metabolite production. This chapter also discusses the hitherto reports on up-scaling of hairy root cultures of various plant species in terms of modifications in designing of bioreactors for incessant tissue growth concomitantly with metabolite production.

Keywords

Agrobacterium rhizogenes · Hairy roots · Bioreactor designing · Fermentor · Scale-up · Secondary metabolites · Root clump · Mass transfer

Abbreviations

ALR	Air lift reactors
BCR	Bubble column reactors
HRCs	Hairy root cultures
NMR	Nutrient mist reactor
STR	Stirred tank

1 Introduction

Biotechnological intercession has triggered tremendous interest in utilizing in vitro culture systems for the production of a variety of promising phytochemicals [1–4]. Majority of phytochemicals are plant secondary metabolites which are commercially important in the form of pharmaceuticals, nutraceuticals, flavors, essential oils, food additives, feed stocks, and antimicrobials. Till date, several biotechnological strategies have been adopted to produce these bioactive secondary metabolites by establishing in vitro cell and organ cultures [5–8]. However, in general, when compared to the intact plants, the production of these secondary metabolites remains at lower side in usual lab-scale cell and organ cultures. Alternatively, scale-up of an in vitro culture by replicating a lab-scale bioprocess in larger culture vessels as closely as possible to produce larger amounts of product may provide a solution for large-scale

production of desired compounds [4, 9]. In vitro cell and organ cultures growing in liquid medium have a unique blend of physical and chemical culture environment and thus easily extend their use in up-scaling in bioreactors. In the same context, a bioreactor is a mechanical device that can simulate particular physio-chemical environment required for incessant growth and physiological activities of cultured cells and tissues. Besides increased working volumes, the semi or fully automated control system and round the year production efficiency that are independent of seasonal or climatic barriers are the additional advantages of up-scaling of an in vitro culture using bioreactors [10, 11]. However, the growth behavior of a biological entity not only depends upon their morphological and physiological status but also influenced by their physical environment. Therefore, successful operation of these bioreactors involve skillful implementation of engineering principles of designing the culture vessels in a way that can significantly influence the growth of cultured tissue at larger volumes [12]. The prime focus during the designing of bioreactor should be on adequate mixing of culture medium with minimized shear stress and optimized mass transfer with reduced hydrodynamic pressure.

Besides microbial cultures, the use of bioreactors has largely been focused on plant cell suspensions and hairy root cultures (HRCs) for secondary metabolite production [13]. Various reports on large-scale culture of cell suspension and hairy roots in various types of bioreactors are available [14–17]. However, genetic instability of cells and their higher sensitivity toward variability in culture conditions limit the broader use of cell suspension cultures for desired metabolite production. Further, many a times, the metabolite production is restricted to differentiated cell/tissues that too at their certain physiological age, and thus, in most cases, culture aging leads to consequent reduced production of desired metabolites. Therefore, keeping in mind the vast potential of HRCs for secondary metabolite production, the up-scaling of these cultures has shown striking opportunities in commercializing a bioprocess for desired metabolite production [18–20]. In up-scaling of hairy root-based production process, there are various factors which greatly influence the production. These include selection of reactor, optimization of culture conditions, automation and interpretation of synergistic effects of various conditions, etc., which needs judicious scientific attention. The upcoming text provides a descriptive account on bioreactor technology for up-scaling of HRCs for secondary metabolite production. The chapter also discuss the hitherto reports on up-scaling of HRCs of various plant species in terms of modifications in designing of bioreactors for incessant tissue growth concomitantly with metabolite production.

2 Hairy Roots as Potential Secondary Metabolite Production System

In the past three decades, hairy roots and secondary metabolite production became synonymous, and plentiful reports have came into existence which reveals various aspects of growth and production potential of HRCs [3, 20–24]. Hairy roots are disease expression of plants that are infected by soil bacterium *Agrobacterium rhizogenes*. The

etiology behind the disease symptoms describes the stable insertion of bacterial T-DNA from extrachromosomal root-inducing (Ri) plasmid into the host plant genome and a consequent possible interruption in host secondary metabolism [25, 26].

Hairy roots being an easy "establish and explore" low-cost culture obligation have progressively materialized into full-fledged global technology for plant-based secondary metabolite production. Miscellaneous beneficial properties like multi-enzyme biosynthetic paraphernalia and close physiological and biochemical similarity with parent plant contribute to the preferential lead for choosing the genetic and biochemically stable HRCs over other in vitro systems for the production of variety of phytochemicals that is commercially important [21, 27]. In addition to such exclusive properties, hairy roots are also unique as in some cases they produce compounds that are not known in normal roots. The examples of production of glycoside conjugates of flavonoids in HRCs of *Scutellaria baicalensis* Georgi and sarpagine alkaloids from *Rauwolfia serpentina* are relevant to mention here [28]. Normal roots of *S. baicalensis* are known to produce glucose conjugates only. Additionally, sometimes HRCs are also known to produce compounds in higher quantities as compared to those that are present in normal intact roots [28].

A primary cause of the lack of in commercial production of secondary compounds using HR culture systems is their low yield. Several strategies, like elicitation, metabolic engineering, etc., that manipulate inherent property of hairy root tissues to enhance their production potential have been adopted to increase the production of bioactive compounds utilizing HRCs [20, 29–31]. Out of these strategies, the bioreactor up-scaling deals with the principle of large-scale metabolite production in proportion of high biomass growth. Succinctly, higher biomass means higher metabolite production [32]. Thus, keeping in mind high biomass production as the main objective, various types of bioreactors have been designed for the cultivation of hairy roots [32, 33]. A continuous culture in bioreactor can sort out the problems related to the manual handling of cultures and thus rescue the growing tissues from possible microbial infection that occur during regular subculturing. All kinds of bioreactors have heterogenous systems comprising of two or more phases of liquid, solid, and/or gaseous phase. The major confront during the operation is to maintain a homogenous condition which assures optimized mass and heat transfer among the different phases [34]. To deal with the situation, a typical bioreactor consists of various probes and devices to monitor and maintain different physical (temperature, flow rates and turbidity etc.) and chemical (dissolve oxygen, pH) conditions (Fig. 1). The following text discusses the challenges during the maintenance of optimized culture conditions for hairy root growth.

3 Major Challenges in Up-Scaling of Hairy Roots in Bioreactors

Although, HRCs have shown tremendous possibilities in secondary metabolite production at large scale; at the same time, there are certain challenges in using bioreactors for their up-scaling [34, 35]. Normally, a typical scale-up that starts in laboratory includes jars or shake flasks of 50–250 ml which further moves to

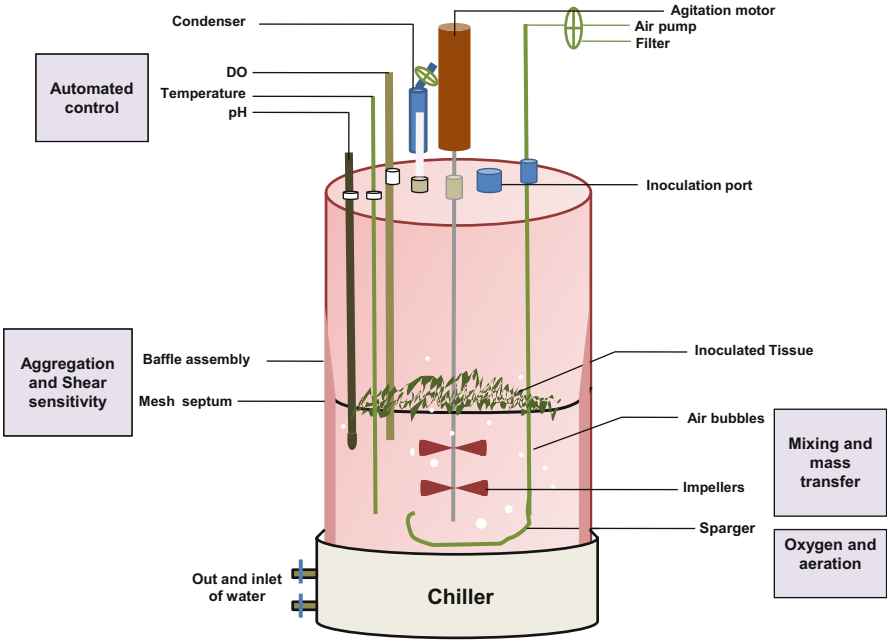

Fig. 1 Schematic presentation of bioreactor configuration

500 ml–10 L small-scale bioreactor. However, for large or industrial scale, stainless steel vessels of varying sizes (>10 L) are required (Fig. 2). It is well known that before going for up-scaling of any culture, optimization of process at bench scale is a prerequisite. However, at this stage, it is relevant to state that the results of bench-scale optimization cannot be directly transferable to higher scales. This is because a large vessel bioreactor may provide totally different culture conditions, and with the increase of vessel size, the homogenous culture ambience remains no longer effective. At this stage various physical factors like gas and liquid flow rates, mass transfer rate, concentration gradients, etc., start affecting the tissue growth simultaneously. These challenges are scientifically handled with the use of proper bioreactor technology that deals with the utilization of engineering principles and mathematical formulations to optimize a bioprocess [20, 36]. At laboratory bench scale, one can only check the practical feasibility of up-scaling of bioprocess in terms of desired productivity and production cost at industrial scale [35]. Various physical and chemical aspects during up-scaling of HRCs that are needed to be judiciously attended are described in upcoming text.

3.1 Aeration, Agitation, and Mixing

The O_2 supplementation in reactor vessel is determine by the air flow supply. The basic objective of continuous O_2 supplementation is to perform all biological

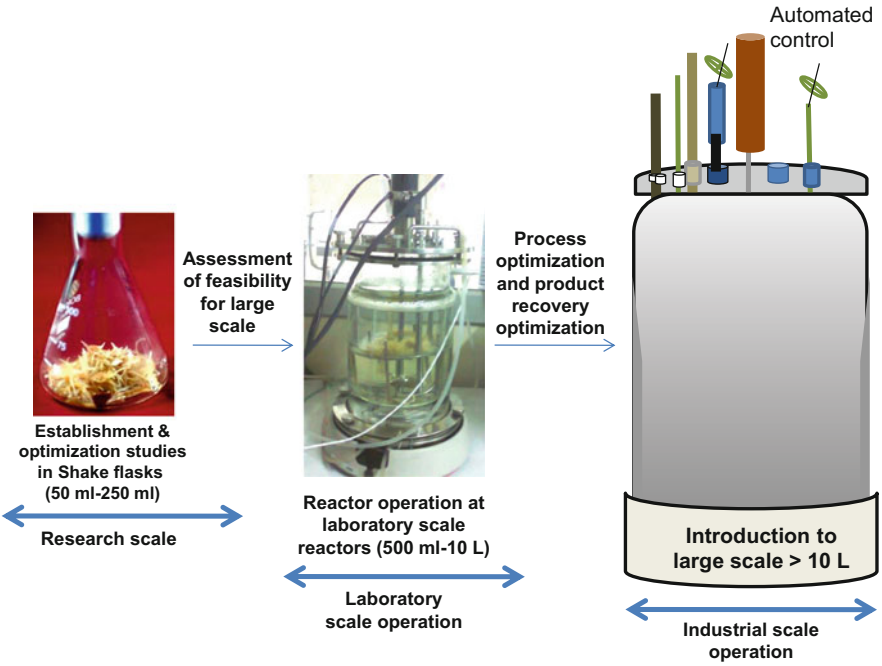

Fig. 2 Gradual process scale-up of culturing hairy roots

activities by growing root tissue, while agitation, on the other hand, assures the homogeneity of liquid medium [37]. The aeration system consists of sparger, while for mass/heat transfer and uniform air distribution, agitator or impellors are required. Keeping in mind the shear sensitivity and bubble tolerance of growing tissue, normally, stainless steel spargers are used to aerate the bioreactor vessel [38, 39]. However, the bubble size and distribution of bubbles within in the liquid significantly influence the gas holdup and unit time for gaseous exchange or mass transfer by the cells in contact. Thus, a judicious attention to the bubble size and distribution improves the understanding of cell behavior under hydrodynamic pressure and mass transfer phenomenon [40]. For this purpose different types of spargers (porous, orifice, etc.) in combination with different types of impellers (turbine or marine blade, propellers, etc.) are used [41]. This designing of reactor vessel depends upon the morphology and growth pattern of hairy roots to be grown. One more point which needs attention during the selection of vessel design is vortex formation. To prevent vortex formation due to continuous mixing, baffles or metal strips attached on radial sides of vessel are used (Fig. 1). In an effort to develop a bioreactor for enhancing the biomass yield of plant organs, the HRCs of *Hyoscyamus muticus* were grown in a 5 L capacity bioreactor of which culture vessel was provided with an elongated baffle assembly [42]. The baffle not only prevented vortex formation but also facilitated rotary transport of radial flow of aqueous medium in the vessel.

Similar reactor was later on successfully used to enhance the biomass yield of HRCs of *Picrorhiza kurroa* [43] HRCs, and multiple shoot cultures of *Glycyrrhiza glabra* [44, 45].

3.2 Root Morphology and Clump Formation

Hairy root cultures of different plant species exhibit differential morphology. Furthermore, the interclonal morphological difference is also significant in hairy root clones of a plant [46]. The randomness of insertion sites of T-DNA into host genome and the number of copies inserted collectively contribute to these interclonal morphological differences. The unique feature of hairy root growth is that they readily form clumps having densely packed rigid central core surrounded by loose highly entangled peripheral root mass [14, 47–49]. The dense mass of root clumps endorses bubble coalescing and channeling, poor liquid mixing, and ultimately the localized liquid stagnation. This hampers the continuous air and nutrient flow to the tissues growing toward the center by the resistance provided by peripheral root mass. This creates a phase difference between liquid (medium) and solid (tissue clump) components of growth matrix. The poor localized intraclump penetration of liquid flow greatly impose nutritional and concentration gradients near and within the clump [50, 51]. Such deficiency may lead to the cell necrosis and death at the clump center. These kinds of limitations can be overcome by applying controlled agitation rate and aeration with appropriate impellers and spargers, respectively [52]. For example, porous polypropylene membrane tubing as a supplementary aeration device was used to directly deliver the O_2 to *Atropa belladonna* hairy root clumps. This was found helpful in overcoming mass transfer resistance related with insufficient intraclump penetration of liquid current and improved the biomass production by 32–65 % higher than sparging only of air [53]. Earlier, in similar context of analyzing the effects of oxygen limitations on *Atropa belladonna* hairy root growth in 250 mL shake flask cultures, oxygen limitations are likely to affect biomass production and kinetic measurements in shake flask cultures of hairy roots [54]. These kinds of optimization and investigation studies at flask level paved the way for acquiring novel strategies for site-directed oxygen delivery into the zones of highest root density in large culture vessels of reactor.

The presence of root hairs also plays an important role in increasing mass flow resistance. Studies reveal that root hairs which improve nutrient uptake under natural conditions are observed unfavorable in liquid medium as they produce resistance to fluid flow and limit the O_2 supply to entangled root masses. In bioreactor cultivation of *H. muticus* transformed root cultures, the root hairs were found as substantial contributor of high fluid flow resistance [55]. To deal with such morphological constraints, some studies proposed the idea of growing hairless roots. In such studies either a root hair mutant line was selected or the root hairs in a normal hairy root clone were chemically removed. In an experimental system, hairy and hairless transformed roots of *H. muticus* were developed to assess the inhibitory role of root hairs in mass transport. Hairless lines of the test plant were initiated using

pyrene butyric acid (PBA) and phosphate. The mixing was significantly enhanced in hairless root cultures grown in a 15 L bubble column reactor. The growth rate of the hairless culture in the bioreactor was as much as 2.4 times greater than growth of the hairy culture under similar conditions. The improved reactor performance was reflected in greater biomass accumulation and respiratory activity. These results show that the root hairs – which facilitate nutrient uptake in a static soil environment – are detrimental to growth in a liquid environment due to their involvement in stagnating fluid flow and limiting oxygen [56, 57].

3.3 Shear Stress and Hydrodynamic Pressure

Effects of hydrodynamic stress on suspended cells and tissues have been the matter of investigation since the beginning of reactor technology. Shear stress is the condition which reflects the extra burden on cell caused by continuous agitation of medium, distribution and fragmentation of gas bubbles due to the stirrer, and bubble rupture at the liquid surface. Hairy roots are also sensitive to hydrodynamic shear. However, no generalized strategy to minimize the shear stress has been concluded as shear stress on hairy roots of a plant species is supposed to be collectively governed by certain factors like tissue morphology, age of culture, aeration and agitation speed, viscosity of liquid medium, etc. Therefore, not only the designing of reactor vessel but also the selection of suitable root line and composition of medium may reduce the shear stress during culture. Although various practical strategies to minimize the shear stress and improve shear tolerance have been discussed for up-scaling of animal cells, for HRCs this aspect has been less explored [58, 59]. Furthermore, mathematical formulations have also been explored to calculate and optimize the shear rate in a continuously operating vessel. Nevertheless, for HRCs and their up-scaling in bioreactors, such studies are sparse and yet to be explored [60].

3.4 Mass Transfer

In multiphase system of bioreactor vessel, mass transfer takes place over phase boundaries. Computational analysis of this mass flux enables to assess the mass distribution in different phases over time and space in the system. The purpose of such computational analysis is to understand the growth behavior of roots and further design as well as control the process [61–63]. The efficient mass transfer between roots and their growth environment may define the growth characteristics of roots. In a case study of *Tagetes patula* hairy roots, methodical testing was performed in order to determine the relative significance of gas-liquid and solid-liquid mass transfer [64]. In shake flask cultures, the biomass growth largely depends upon the volume of medium and shaker speed. Taking an insight, this dependency of root growth indicates the importance of mass transfer in relation to different volumes of culture medium and shaker speed. Such calculation for a specific hairy root line

also makes the basis for the selection of suitable bioreactor for up-scaling of that root line.

The gradient in chemical composition of phase boundaries, which occur due to continuous utilization of O_2 by rapidly growing tissue, is responsible for the driving force for mass transfer. Several studies have been performed to investigate the O_2 requirement and mass transfer phenomenon in HRCs [21, 54, 65]. Taking *A. belladonna* as model hairy root culture, convection was found to be the dominant mechanism for mass transfer in dense areas of root growth [65]. The mass transfer phenomenon was observed through local dissolve O_2 level and rate of O_2 uptake at the vessel areas where roots are loosely and/or densely packed in clumps. Specific growth rate and an exponential increase in root growth in terms of root length and number of growing root tips were observed at oxygen tensions between 70 % and 100 % air saturation. As the O_2 supplementation decreases by 50 %, the root growth got negatively affected specially in the areas where root tissues have formed dense clumps. Due to the dense entangled mass, the air saturation at such points decreased by half or even low when compared to the areas where roots are loosely packed [65]. Such results indicated mass transfer resistances near the gas-liquid and liquid-solid boundary layer which dominantly affect the oxygen delivery to the growing hairy roots.

In the past few years, computational and mathematical simulations have been used to model the mass transfer behavior in bioreactors for hairy root growth [66, 67]. In a study, a computational fluid dynamics (CFD) model was developed to simulate the hydrodynamics and oxygen mass transfer in hairy roots growing in bioreactor. The CFD model predicted increase in mass transfer rates in root clumps that are stimulated by ultrasound. The model predictions were validated through experimental results in which increased O_2 transfer was observed. This increased O_2 was correlated with increased membrane permeability of root tissues by ultrasound stress [67]. Similarly, in another study a mathematical model was developed to investigate O_2 transfer kinetics in *Azadirachta indica* hairy roots [68]. The model simulates and predicted the onset of O_2 transfer limitation in dense intraclonal areas which eventually indicated the need of increased O_2 supply to prevent the subsequent inhibition in growth of the hairy root biomass due to oxygen transfer (diffusional) limitation. Thus, computational/mathematical simulations of mass transfer phenomenon in a hairy root-based bioprocess may help in monitoring and controlling the process.

3.5 Optimization of Process: Medium Components, Temperature, Light, pH, and Inoculation

Chemical composition of nutrient medium directly influences the biomass yield and productivity of metabolites in HRCs. Therefore, studies on the effect of preferred key medium components on tissue growth, as well as product accumulation for incessant productivity, are a primary requisite. Further, this is also crucial to estimate secondary metabolite production as conditions suitable for growth may sometimes adversely affect the product formation and vice versa. Besides the conventional

method of growth medium optimization which includes the alteration in various components individually and observing their effect on tissue growth, nowadays for the optimization of various growth conditions, the computational modeling is observed as a flexible strategy [69, 70]. Recently, the use of artificial neural networks (ANN) alone or in combination with other mathematical concepts was proposed to optimize various physical and chemical culture conditions for desired productivity in HRCs [70]. These studies pave the way for comparisons and simulations of similar bioprocess at larger levels involving bioreactors. With particular reference to the scale-up cultures using large reactor vessels, the computational simulations of growth under varying chemical conditions provide results mostly near to accuracy. Besides, this also helps to overcome the cost and time overflow of the conventional optimization. Spatial variations in culture temperature and medium pH lead to variations in physiological performance of the cultured roots. Continuous effect of light and temperature variation on growth and metabolite production has been observed in hairy roots of *Catharanthus roseus*, *Artemisia annua*, *Echinacea purpurea*, and *Panax ginseng* [71–75]. In *P. ginseng* hairy roots growing in 5 L bioreactor setup, biomass accumulation and ginsenoside production were found highest under red light fluorescence [75]. Therefore, optimization of the physical parameters for best results of hairy root up-scaling in bioreactors is as important as other factors. Though rather much explored in microalgae culture, the present hype in hairy root biotechnology has shown the viewpoint of using photobioreactors for large-scale culture of light-sensitive roots [76]. In case of light-sensitive roots, formation of clump leads to uneven distribution of light. Therefore, the basic principles for developing a reactor for such root cultures must include efficient and quantitative understanding of both mass and light transfer. On the other hand, the temperature of the liquid medium can easily be adjusted by circulating water in a chiller jacket outside the glass vessel.

Another aspect of process optimization that requires proper attention is the inoculation of tissue for scale-up. This not only includes optimization of inoculum density-media volume ratio and the selection of explant material of desired physiological age but also the aseptic procedure through which transfer of tissue from smaller culture container to reactor culture vessel is done. The former is done by subculturing the hairy roots in shake flasks and allowing them to grow till their logarithmic/exponential phase begins. At the onset of exponential phase, the root tissues that are well adapted to fast growth are aseptically transferred to the reactor vessel through the inoculation port of the bioreactor. Expert handling and incessant maintenance of aseptic conditions are the prerequisites of the inoculation procedure which is done under sterile laminar hood. Sometimes, as an extra precautionary measure during inoculation, the inoculation port is optimally flamed with the sterile cotton swabs soaked in alcohol [43]. Further, in an experiment, alginate-encapsulated *R. serpentina* hairy root tips were used as inoculum for the reactor vessel (unpublished results). This made the inoculation procedure a bit easier as simple pouring of small amount of liquid medium containing beads was required rather than transferring the whole root tissue using sterile forceps. However, pragmatic results of a detailed study on growth performance of alginate-encapsulated hairy root tips of

R. serpentina were behind the idea of using encapsulated tips as inoculum of bioreactor [46]. Thus, a prior knowledge of growth performance of alginate hairy root tips of other plants is required before using them as explants for inoculating the bioreactor.

4 Overcoming the Challenges: Designing of Bioreactor for Hairy Root Cultures

By definition, a bioreactor is a culture vessel used for any biological conversion in terms of cellular growth and related productivity. These conversions are either in the form of cells/tissues/organisms cultured in a set of defined conditions or chemical compounds that are converted or transformed through specific metabolic reactions by mediation of a biological entity. The only difference between bioreactors and conventional chemical reactors is that the former is specifically used to culture biological entities. Another term that is used parallel to bioreactor is "fermenter" which is strictly used for anaerobic processes. Bioreactor designing is an engineering practice which inroads to a field that is known only for biology-based phenomenon. Thus, bioreactor designing characterizes an amalgamation of engineering principles of designing and analysis into growth and production processes of biological entities. The key issue in designing and operation of bioreactor is to control a biochemical phenomenon for a defined period in a consistent optimized way to get incessant maximum productivity. This optimization can be done on two scales; first, the biological entity and its products which include physiological basis of cell line/clone selection, metabolite synthesis, and accumulation, etc. The second scale of optimization includes physical parameters of culture vessel like temperature, pH, air supply, medium continuity, product removal, etc. The second scale basically includes designing a reactor vessel in such a way that maximum physical parameters of culture remain optimized and function in equilibrium to result in desired and consistent productivity. Several reports on cultivating hairy roots in large culture vessels endorse the idea of designing to facilitate adequate O_2/nutrient supply during the entire culture duration [32, 33, 77].

On the basis of mode of operation, the bioreactor culture can be batch culture, continuous culture, and semi-continuous culture. Selection of bioreactor operation for hairy root cultivation depends upon various objectives. With particular reference to the HRCs, the bioreactor technology follows the concept of proportionate production of biomass and respective metabolites and, thus, can also be considered as a potential yield enhancement strategy [20]. Initially, Rhodes et al. [47] published a successful report on *Nicotiana rustica* HRCs in a bioreactor for nicotine production. However, during the past two decades, the bioreactor technology for HRCs has significantly uplifted from laboratory bench scale to industrial scale as various companies are now ready to adapt modified versions of technology to cultivate hairy root biomass for metabolite production [16; ROOTec bioactives Ltd, Switzerland; http:// www.rootec.com; CBN Biotech, South Korea]. Nevertheless, the key challenge in commercialization of bioreactor technology for HRCs is the low

productivity of cultures and overall cost of technology [16, 48]. Persistent inflow of ideas can be observed in various reactor configurations that have been advised time to time to overcome limitations of low productivity like shear stress, heterogeneity, mass transfer, and nutrient uptake [32]. A detailed account on various types of bioreactors designed to cultivate HRCs has been given in Table 1 and Fig. 3. Such designing of reactors vessels is made to achieve the major objectives like its suitability to the morphology and physiology of tissue to be grown, adequate optimized culture conditions for maximum productivity, and finally the cost of the procedure. The reactors used to cultivate hairy roots can be divided into liquid phase, gas phase, and hybrid reactors.

4.1 Liquid Phase/Submerged Reactors

In liquid-phase reactors, HRs are allowed to grow throughout in liquid ambience comprised of growth medium. As the roots always grow under submerged conditions, liquid-phase reactors are also known as submerged reactors. Stirred tank (STR) and air lift (ALR) are the simple submerged reactors which are used to grow hairy roots. The STRs consist of impeller to facilitate mass transfer to the growing root tissue. In an earlier study, STR was used to grow HRCs of *Datura stramonium* [78]. However, the major challenge in these reactors is shearing and wounding of root tissues due to continuous rotation of impeller blades. Besides, in compactly grown root culture, poor liquid circulation and chemical gradients also contribute to the insufficiency of these reactors [79]. To deal with such challenges, the use of steel mesh or any porous substrate made up of nonreactive material was proposed [78]. HRCs of *Glycyrrhiza glabra*, *Rauwolfia serpentina*, and *Picrorhiza kurroa* were successfully grown in these modified STRs where a nylon mesh (pore size 200 μ) was provided to avoid submergence of inoculated tissue and its damage from the impeller [43, 44, 46]. Further, the idea of using of reactors without any mechanical agitation came into existence, out of which air lift and bubble column reactors are the most common examples. In air lift reactors, at the bottom of the vessel, aerators are provided that supply compressed air moisten with liquid medium at slow rates. The air lift reactors were used for HRCs of *Lithospermum erythrorhizon*, horse radish, and carrot [48, 81]. Further, *Solanum chrysotrichum* HRCs were also grown separately in a 2L air lift reactor with basic design and a novel modified mesh-draught reactor [82]. The tissue growth and distribution within the vessel was found more desired in later as in continuous operation, the insufficient mixing and nonuniform nutrient supply to the rapidly growing root tissue mass in air lift reactors led to the limiting factors for growth.

The much similar to air lift, bubble column reactors (BCRs) are designed to provide air in the form of bubbles to the liquid medium where the roots are submerged. In these reactors the rate of gaseous supply increased gradually with the tissue growth. Several studies have been performed on growing hairy roots using bubble column reactors with little attachments and modifications. For example, in the case of *Tagetes patula* and *Lithospermum erythrorhizon* HRCs, the division of bubble column into two segments with sparger in each segment was done to increase the mass transfer as a whole [83, 84]. Successful use of bubble column reactor is also

Table 1 Comparative account on various major types of bioreactor used for up-scaling of hairy root cultures

Type of reactor	Bubble column (BCR)	Stirred tank (STR)	Air-lift (ALR)	Convective flow (CFR)
Features and modifications	Vertical cylindrical column in which gas is inserted from the bottom in the form of bubble Can be segmented or non-segmented Mesh septum can be provided for anchorage	Fitted with impellers that facilitates mass transfer Mesh septum can be provided for anchorage and to avoid tissue damage from impeller blades	Glass grid / sparger fitted at the bottom is used to provide compressed air. Different from BCRs as they contain a draft tube which improves circulation and oxygen transfer and equalizes shear forces in the reactor	STRs attached to a tubular culture chamber having support mesh at the lower end Mass transfer occurs by means of convective medium flow around growing tissue Air supply takes place by means of external aeration vessel
Advantages	Low capital costs Simple mechanicalconfiguration Low energy requirements	Ideal for continuous operation Better mass transfer in comparison to BCR	Simple design with no moving parts or agitator for less maintenance Air lifts give more vigorous recirculation for the same air flow	Allows oxygen rich medium to flow simultaneously through external aeration
Disadvantages	Not suitable for viscous liquids Enlargement and Entrapping of bubbles in root clumps, resulting in gas flow channeling around clumps and total localized depletion of oxygen	High possibility of tissue damage More energy consumption Insufficient control of concentration gradients near dense root clumps	Greater air throughput and higher pressures needed. May cause excessive foaming and requires more energy for their generation at porous distributors	May not be a realistic large-scale system due to the pressure required to circulate the culture medium at a velocity high enough to overcome the flow resistance of the root bed
References	[32, 83–85]	[32, 48, 78, 80, 107]	[48, 80–82, 108, 109]	[55]

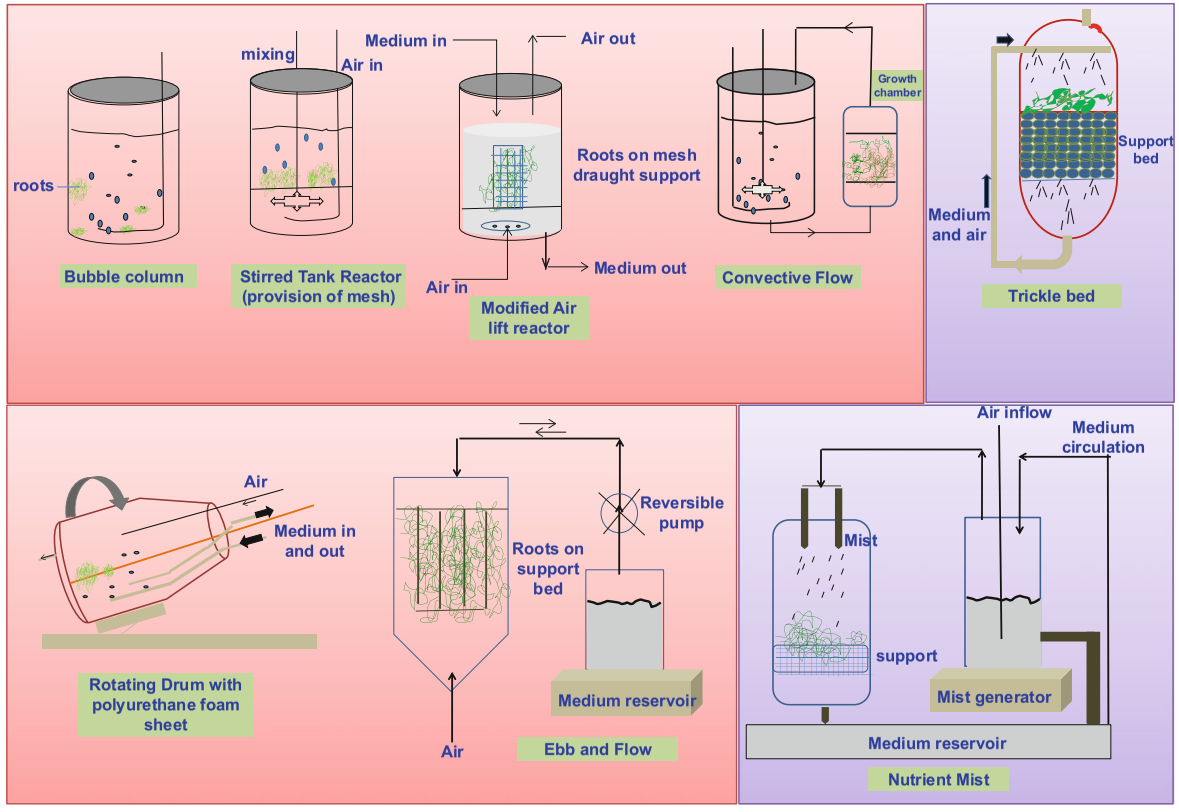

Fig. 3 Schematic presentation of various types of major liquid (red background) and gas phase (blue background) bioreactors used for up-scaling of hairy root cultures

reported for betalain production from HRCs of *Beta vulgaris* challenged with different elicitors [85]. However, the betalain productivity was hampered because of its inhibitory action on biomass. In an extension of this, cetyl trimethyl ammonium bromide (CTAB) was used for pigment release which resulted in optimization of concentration for better efflux of betalains without showing any inhibitory effect on hairy root viability. These studies on product enhancement and online extraction of pigment are useful for developing a bioreactor system for specific hairy root cultures. In particular the use of elicitors and efflux studies provide an insight for integrating unit operations and developing a process for continuous operation and higher production of phytochemicals.

Acquiring the concept of mass transfer based on convective fluid flow, convective flow reactors (CFR) were designed for HRCs of *Hyoscyamus muticus* [55]. The reactor consisted of a stirred tank along with a separate tubular culture chamber for root growth. The medium circulate through the two chambers at a velocity sufficient to overcome the flow resistance of the dense root bed. In a comparison with BCR, the *H. muticus* hairy root growth in CFR exhibited enhanced biomass production [55]. Proportionate to tissue growth, the sufficient medium supplementation at optimized velocity contributed enhanced convective mass transfer subsequently led to the enhanced growth. In other studies, to assess the effect of liquid flow velocity on the growth, oxygen uptake and productivity of hairy roots single column CFR were developed for *Beta vulgaris* [86]. In this study, the optimal range of velocity for good growth was observed 15 mh^{-1} and the increased flow velocity up to 28 mh^{-1} though enhanced the root elongation rate, but the viability of root tips was reduced due to damage from shear stress.

Based on this result, a radial flow reactor was developed to minimize the pressure on root tissue surface created by the fluid flowing in at a specific velocity. A substantial increase in biomass was observed in this reactor configuration where air-saturated medium flow in at a flow velocity 15 mh^{-1} through the ports on the sidewall of the reactor and flow out through the ports at the center of the top and bottom plates. Likewise, *Atropa belladonna* and *Solanum aviculare* [63] HRCs were also grown in packed bed recirculation reactor to analyze the liquid solid hydrodynamic layer and oxygen uptake. In the experiment, the liquid medium was flown in high velocity to minimize the hydrodynamic boundary layers around the tissue surface. However, it was observed that the rate of oxygen uptake and growth by the roots was dependent on the tissue mass, and very thin or the absence of hydrodynamic boundary layer did not affect the growth. It was proposed that under submerged conditions, roots were covered with thick layers of hydrated mucilage which acts as an additional barrier to oxygen transfer.

Keeping such observations in mind, the idea of cultivating hairy roots in alternate fill and drain cycles of liquid and gas phase has came into existence. To materialize this concept, rotating drum (RDR) and ebb and flow reactors (EFBR) were developed [48, 49, 80]. Rotating drum consists of a drum-shaped vessel mounted on rollers for rotation at low speed of 2–6 rpm. The difficulty observed with the rotating drum reactor was that at the beginning of culture, roots did not adhere to the vessel wall. Consequently, as the roots were rotated above the culture medium, they

detached from the wall and were damaged ultimately resulting in low productivity. For the cultivation of carrot hairy roots, Kondo et al. [80] used polyurethane foam to immobilize the roots to overcome this problem. Though such modification had solved the challenge up to much extent, the RDRs were found unsuitable for industrial scale as they require high energy consumption [87]. On the other hand, the EFBR derived its name from the process behavior of liquid medium in reactor which follows repetitive cycles of ebbing and flowing (filling and draining) between the reactor vessel and medium reservoir [51, 88, 89]. Designed for *H. muticus* HRCs, the EFBRs demonstrated successful scale up to 50× of a 50 ml flask. In a comparative study with bubble column reactors, the EFBRs were found superior in terms of productivity, and liquid holds up profiles by dense entangled root mass. The study concluded that it was back and forth convective flow of EFBRs that made it superior to bubble columns which have negligible fluid convection.

Based on the concept of temporary contact between the cultured tissue and liquid medium, temporary immersion systems (TIS) were also used for the cultivation of hairy roots. These systems allow the cultured tissue to be immersed in liquid medium for a defined duration (flooding) followed by standby stage. Basically, TIS are designed for in vitro plant propagation; however certain advantages of these systems, like ease of medium changes, limited shear damage, less chances of hyperhydration of tissue, etc., have attracted researchers to use them for hairy roots [90]. In a study of *Beta vulgaris* HRCs, RITA® (Recipient for Automated Temporary Immersion System) apparatus was used to grow hairy roots for betalains biosysnthesis [91]. The maximum accumulation of betalain pigment was observed with 15 min immersed/60 min standby cycles, whereas optimal biomass in terms of intensively branched and morphologically healthy roots was obtained with 15 min immersed/75 min standby cycles. Thus, this was observed as an advantage of TIS in root growth in comparison to the ALR, STR, and bubble column reactors where clump formation and shear damage followed by nutrient and oxygen limitations in root tissues at central core and callus formation, respectively, are associated with loss of productivity. Forced and repeated air replenishment in the system and lesser hydrodynamic pressure to the growing tissue are supposed to be the reasons behind optimal growth of the root tissues in these systems. However, the success of using temporary immersions depends upon (1) the ratio of inoculum density and medium volume that too in relation with the size of container and (2) the optimization of length and frequency of medium immersions. In TIS, the time and frequency of the immersion are the most decisive parameter for optimized productivity, as they influence nutrient and water uptake. Thus, a prior optimization of such parameters is a must requisite. Another point of limitation that would be in need of attention is that, normally, TIS are smaller in size with small interior space which may prove insufficient for fast-growing root tissues.

4.2 Gas-Phase Reactors

In gas-phase reactors, the roots are immobilized in culture vessels with the help of horizontal sheets or rings made up of inert material and exposed to air or a gas

mixture. The liquid medium is provided in the form of spray or mist of micron-sized droplets. In such reactors liquid is the dispersed phase and gas is the continuous phase in which roots are exposed. Such setup diminishes many of the limitations associated with liquid-phase reactors. Out of various gas-phase rectors, trickle-bed reactors (TBR), nutrient mist rectors (NMR), and droplet phase are the common reactors. Since the medium is supplied in the form of droplet, there is a considerable variation in the size of droplets according to the requirement, and various attachments, like ultrasonic transducers, spray nozzles, etc., are used to create perfect sized droplets ranging from 0.5 to 50 μm [62, 92, 93]. In trickle-bed reactors, the medium trickles over roots from the top of the vessel. The used up medium is drained from the bottom of the vessel to a pool and is recirculated at a specific rate. The flow of liquid depends upon gravity and the distribution of liquid depends upon the mechanism of liquid delivery from the top. The growth of *H. muticus* hairy roots in gas-dispersed reactor exhibited excellent performance and accumulated tissue mass in submerged air-sparged reactors was 31 % of liquid-dispersed controls [49]. A study concluded that the distribution of the roots becomes a key factor in controlling the rate of growth. Noticeable results for hairy root growth, fluid dynamics, and oxygen mass transfer in a trickle-bed reactor were also obtained from the study conducted by Ramakrishnan and Curtis 2004 [94]. These results demonstrate that trickle-bed reactor systems can sustain tissue concentrations, growth rates, and volumetric biomass productivities substantially higher than other reported bioreactor configurations. Mass transfer and fluid dynamics are characterized in trickle-bed root reactors to identify appropriate operating conditions and scale-up criteria. Bioreactor characterization is sufficient to carry out preliminary design calculations that indicate scale-up feasibility to at least 10,000 L. On the other hand, in mist reactors, mists are more water efficient, thus, eliminating the need for extensive recirculation equipment. In comparison to large droplets of spray, provision of mists facilitates nutrient and gaseous exchange by reducing the thickness of the liquid film deposition on the surface of the root tissue [62, 95, 96]. Irrespective of spray or mist, the liquid that forms thin layer on the tissue surface acts as a barrier for efficient mass transfer [96, 97]. On the other hand, roots in a mist reactor are often too sparsely packed to capture mist particles efficiently and cannot, therefore, meet the nutrient demands required to maintain high growth rates. Keeping this in mind, an aerosol model was proposed for *Artemisia annua* hairy roots growing in NMR [98]. Growth rate was increased when mist medium containing high sucrose concentration was used in which sucrose acted as aerosol particles. In another recent study, an ON/OFF strategy was analyzed for optimizing the operating conditions of a mist reactor for the growth of hairy roots [99, 100]. A mathematical model was developed to optimize the ON/OFF mist duty cycle for the specified growth of hairy roots. Considering the availability and rate of transport of nutrients to the roots as vital parameters for growth, the ON/OFF cycles were proposed as the thin liquid film which continuously builds up during the ON cycle over the root surface is a key limiting factor mass transfer. However, the same film can also act as a finite reservoir of nutrients in the absence of any replenishment during the OFF cycle.

Since the continuous phase is gas, the roots are required to be immobilized in the reactor. For the immobilization of roots, various equipments like horizontal or vertical mesh sheets or rings made up of nylon or stainless steel [63, 101–105] were used. Ramakrishnan and Curtis [61] used Intalox metal process packing elements to immobilize roots in gas-phase reactors.

4.3 Hybrid Reactors

Similar to liquid-phase reactors, in gas-phase reactors the major problem includes liquid channeling and holdup within the root bed [61]. The stagnant holdup of liquid may have different nutrient levels than the bulk fluid [106] or make the roots within effectively submerged and depleted of oxygen [107]. To overcome these limitations, hybrid reactors for hairy root cultures are designed which follow the concept of growing roots under submerged conditions initially so that roots get adapted to the process and start growing. After an initial growth when submerged condition no longer remains effective due to dense growth and poor mass transfer rate, the reactor operation switches over to gas phase. Ramakrishnan and Curtis [61] proposed a combination of bubble column and trickle bed for *Hyoscyamus muticus* roots. In the study, bubble column was initially inoculated with roots to allow them to evenly distribute and attach to the anchorage provided in the reactor. Following 2 weeks of growth, when roots started forming clumps, the reactor was switched to a trickle-bed operation for another 4 weeks, thus exposing roots to a gas environment. In another study, *Datura stramonium* hairy roots were grown initially under submerged phase for 21 days followed by droplet phase for 40 days [93]. A hybrid reactor system made up of bubble column and nutrient mist bioreactor was used to study the transient growth characteristics and nutrient utilization rates of *Artemisia annua* hairy roots [108].

5 Conclusion

Hairy root culture technology has offered perspectives for in vitro large-scale production of valuable plant secondary metabolites. Together it also provides many challenges during large-scale cultivation as with the increase in medium volume, vessel size, and culture density, various physical factors, like gas and liquid flow rates, mass transfer rate, concentration gradients, etc., start affecting the tissue growth simultaneously. Besides, the unusual rheological properties and growth patterns of HRCs also contribute to challenges in their up-scaling. This has laid the background of acquiring and investigating novel approaches of bioreactor designing and process optimization. Optimized mass transfer concomitant with low shear stress and hydrodynamic pressure on growing root tissue is the utmost objective of designing a reactor for desired growth and productivity. The selection of right configuration of reactor vessel also depends upon the morphological and physiological properties of hairy roots. Further, a major issue of the commercial

use of a bioreactor setup for hairy root cultivation deals with low capital cost. Keeping this in mind nowadays, bioreactor technology utilizes computational and mathematical simulations for incessant operation. These simulations provide help in meeting the challenges on issues like optimization of physical, biological, and chemical culture conditions, offline and/or online measurement of growth, mass transfer behavior, synergistic effects of various physical and chemical parameters on growth, downstream processing (intracellular/extracellular), product recovery, etc. However, online measurement of biomass growth is a difficult but perennial requirement during bioreactor cultivation. Precise measurement of growth is difficult due to the nonhomogenous nature of cultured tissue. Nevertheless, for offline measurement there are different methods like packed cell volume (PCV), fresh and dry weight estimation, etc. Growth estimation with these methods is relatively time consuming and also require frequent sampling from the running culture at a threat of contamination. Additionally, withdrawing large sample volumes may become problematic while measuring overall productivity against medium volume and inoculum density. To avoid these problems, online measurement is rather observed as an easy and noninvasive method of growth. These include utilization of artificial neural network and response surface methodology-based computational scheming of growth based on variables in culture conditions and predictions of final productivity [109]. Further, measurement of medium conductivity and osmolarity are the other methods that are being used for the growth measurement [110]. Moreover, online fluorescence measurement of metabolic activities of cultured tissues and online monitoring of cell growth by conductometry are the methods that cannot only predict the final biomass at the end of culture but also become helpful in optimizing various parameters during the culture duration [111, 112]. Although such measurement studies have been much explored in cell suspension and microbial cultures, an insight and proper retrieval of information from these studies may provide help in online growth monitoring and up-scaling of hairy root cultures in bioreactor. This may fairly help in filling the gap between capital cost and the benefits of technology at industrial scale.

References

1. Verpoorte R, Contin A, Memelink J (2002) Biotechnology for the production of plant secondary metabolites. Phytochem Rev 1:13–25
2. Rao SR, Ravishankar GA (2002) Plant cell cultures: chemical factories of secondary metabolites. Biotechnol Adv 20:101–153
3. Georgiev MI, Agostini E, Ludwig-Muller J, Xu J (2012) Genetically transformed roots: from plant disease to biotechnological resource. Trends Biotechnol 30:526–537
4. Hussain S, Sheeba F, Ansari S, Rahman A, Ahmad IZ, Saeed M (2012) Current approaches toward production of secondary plant metabolites. Pharm Bioallied Sci 4:10–20
5. Ravishankar GA, Rao RS (2000) Biotechnological production of phytopharmaceuticals. J Biochem Mol Biol Biophys 4:73–102
6. Martin EK, Vishal G, d Susan RC (2008) Pharmaceutically active natural product synthesis and supply via plant cell culture technology. Mol Pharm 5:243–256
7. Karuppusamy S (2009) A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ and cell cultures. J Med Plants Res 3:1222–1239

8. Vijayasree N, Udayasri P, Aswanikumar VVY, RaviBabu Y, Varma M (2010) Advancements in the production of secondary metabolites. J Nat Prod 3:45–51
9. Georgiev MI, Eibl R, Zhong JJ (2013) Hosting the plant cells in vitro: recent trends in bioreactors. Appl Microbiol Biotechnol 97:3787–3800
10. Gerth A, Schmidt D, Wilken D (2007) The production of plant secondary metabolites using bioreactors. Acta Hortic 764:95–104. doi:10.17660/actahortic.2007.764.11
11. Mamun NHA, Egertsdotter U, Aidun CK (2015) Bioreactor technology for clonal propagation of plants and metabolite production. Front Biol 10:177–193
12. Curtis WR (2005) Application of bioreactor design principles to plant micropropagation. Plant Cell Tissue Organ Cult 81(3):255–264
13. Eibl R, Eibl D (2008) Design of bioreactors suitable for plant cell and tissue cultures. Phytochem Rev 7:593. doi:10.1007/s11101-007-9083-z
14. Choi YE, Kim YS, Paek KY (2006) Types and designs of bioreactors for hairy root culture. In: Plant Tissue Culture Engineering; Focus on Biotechnology. Springer, Netherlands vol 6. pp 161–172
15. Barbara R, Pistelli L, Bertoli A, Pistelli L (2010) Plant cell cultures: bioreactors for industrial production. Adv Exp Med Biol 698:203–221
16. Curtis WR (2010) Hairy roots, bioreactor growth. In: Flickinger MC (ed) Encyclopedia of industrial biotechnology. Wiley, New York, pp 1–20
17. Stiles AR, Liu CZ (2013) Hairy root culture: bioreactor design and process intensification. In: Biotechnology of hairy root systems, vol 134 of the series Advances in biochemical engineering/biotechnology. Springer Berlin Heidelberg, pp 91–114, doi:10.1007/10_2013_181
18. Sevon N, Oksman-Caldentey KM (2002) Agrobacterium rhizogenes mediated transformation: root cultures as a source of alkaloids. Planta Med 68:859–868
19. Georgiev MI, Pavlov AI, Bley T (2007) Hairy root type plant in vitro systems as sources of bioactive substances. Appl Microbiol Biotechnol 74:1175–1185
20. Mehrotra S, Srivastava V, Rahman LU, Kukreja AK (2015) Hairy root biotechnology – indicative timeline to understand missing links and future outlook. Protoplasma 252 (5):1189–201. doi:10.1007/s00709-015-0761-1
21. Shanks JV, Morgan J (1999) Plant hairy root culture. Curr Opin Biotechnol 10:151–155
22. Guillon S, Trémouillaux-Guiller J, Pati PK, Rideau M, Gantet P (2006) Hairy root research: recent scenario and exciting prospects. Curr Opin Plant Biol 9:341–346
23. Georgiev MI, Jutta LM, Bley T (2010) Hairy root culture: copying nature in new bioprocesses. Med Plant Biotechnol: 156–175
24. Ono NN, Li T (2011) The multiplicity of hairy root cultures: prolific possibilities. Plant Sci 180:439–446
25. Chandra S (2012) Natural plant genetic engineer *Agrobacterium rhizogenes*: role of T-DNA in plant secondary metabolism. Biotechnol Lett 34:407–415
26. Chandra S, Chandra R (2011) Engineering secondary metabolite production in hairy roots. Phytochem Rev 10:371–395
27. Giri A, Narasu ML (2000) Transgenic hairy roots recent trends and applications. Biotechnol Adv 18:1–22
28. Sheludko Y, Gerasymenko I (2013) Biosynthetic potential of hairy roots for production of new natural products. In: Chandra S et al (eds) Biotechnology for medicinal plants. Springer, Berlin/Heidelberg. doi:10.1007/978-3-642-29974-2_10
29. Mehrotra S, Rahman LU, Kukreja AK (2010) An extensive case study of hairy-root cultures for enhanced secondary-metabolite production through metabolic-pathway engineering. Biotechnol Appl Biochem 56:161–172. doi:10.1042/BA20100171
30. Goel MK, Mehrotra S, Kukreja AK (2011) Elicitor-induced cellular and molecular events are responsible for productivity enhancement in hairy root cultures: an insight study. Appl Biochem Biotechnol 165:1342–1355
31. Zhou ML, Zhu XM, Shao JR, Tang JR, Wu YM (2011) Production and metabolic engineering of bioactive substances in plant hairy root culture. Appl Microbiol Biotechnol 90:1229–1239

32. Kim YJ, Wyslouzil BE, Weathers PJ (2002) Secondary metabolism of hairy root cultures in bioreactors. In Vitro Cell Dev Biol Plant 38:1–10
33. Srivastava S, Srivastava AK (2007) Hairy root culture for mass production of high-value secondary metabolites. Crit Rev Biotechnol 27:29–43
34. Martin Y, Vermette P (2005) Bioreactors for tissue mass culture: design, characterization, and recent advances. Biomaterials 26(35):7481–7503
35. Pörtner R, Nagel-Heyer S, Goepfert C, Adamietz P, Meenen NM (2005) Bioreactor design for tissue engineering. J Biosci Bioeng 100(3):235–245
36. Chen H, Hu Y (2006) Bioreactors for tissue engineering. Biotechnol Lett 28:1415. doi:10.1007/s10529-006-9111-x
37. Chisti Y, Jauregui-Haza UJ (2002) Oxygen transfer and mixing in mechanically agitated airlift bioreactors. Biochem Eng J 10:143–153
38. Moo-Young M, Chisti Y (1988) Considerations for designing bioreactors for shear-sensitive culture. Biotechnology 6(11):1291–1296
39. Chisti Y (1999) In: Flickinger MC, Drew SW (eds) Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation. Shear sensitivity, vol 5. Wiley, New York, pp 2379–2406
40. Chisti Y, Moo-Young M (1988) Hydrodynamics and oxygen transfer in pneumatic bioreactor devices. Biotechnol Bioeng 31:487–494
41. Junker BH, Stanik M, Barna C, Salmon P, Buckland BC (1998) Influence of impeller type on mass transfer in fermentation vessels. Bioprocess Eng 19:403–413
42. Banerjee S, Kukreja Arun Kumar, Verma PC, Kahol AP, Kumar S (2003) Bio-reactor for enhancing the biomass yield of plant organs. US 6589780 B2
43. Verma PC, Singh H, Negi AS, Saxena G, Rahman LU, Banerjee S (2015) Yield enhancement strategies for the production of picroliv from hairy root culture of *Picrorhiza kurroa* Royle ex Benth. Plant Signal Behav 10(5):e1023976
44. Mehrotra S, Mishra BN, Kukreja AK (2009) Efficiency of liquid system for mass multiplication of *Glycyrrhiza Glabra* and evaluation of genetic fidelity of micropropagated plants. Int J Biotechnol Biochem 5:157–169
45. Mehrotra S, Kukreja AK, Khanuja SPS, Mishra BN (2008) Genetic transformation studies and scale up of hairy root culture of *Glycyrrhiza glabra* in bioreactor. Elect J Biotechnol 11(2):1
46. Mehrotra S, Goel MK, Srivastava V, Rahman LU (2015) Hairy root biotechnology of Rauwolfia serpentina: a potent approach for the production of pharmaceutically important terpenoid indole alkaloids. Biotechnol Lett 37:253–63. doi:10.1007/s10529-014-1695-y
47. Rhodes MJC, Hilton MG, Parr AJ, Robins RJ (1986) Nicotine production by 'Hairy Root' culture of nicotiana rustica: fermentation and product recovery. Biotechnol Lett 8:415–420
48. Taya M, Yoyama A, Kondo O, Kobayashi T (1989) Growth characteristics of plant hairy roots and their cultures in bioreactors. J Chem Eng Jpn 22:84–89
49. McKelvey SA, Gehrig JA, Hollar KA, Curtis WR (1993) Growth of plant root cultures in liquid and gas dispersed reactor environments. Biotechnol Prog 9:317–322
50. Tanaka H (2000) Technological problems in cultivation of plant cells at high density. Biotechnol Bioeng 67:775–790
51. Cuello JL, Yue LC (2008) Ebb-and-flow bioreactor regime and electrical elicitation: novel strategies for hairy root biochemical production. J Integr Biosci 3(1):45–56
52. Carvalho EB, Holihan S, Pearsall B, Curtis WR (1997) Effect of root morphology on reactor design and operation for production of chemicals. In: Doran P (ed) Hairy roots. Gordon and Breach Reading, UK, pp 151–167
53. Kanokwaree K, Doran PM (1998) Application of membrane tubing aeration and perfluorocarbon to improve oxygen delivery to hairy root cultures. Biotechnol Prog 14:479–486
54. Kanokwaree K, Doran PM (1997) The extent to which external oxygen transfer limits growth in shake flask culture of hairy roots. Biotechnol Bioeng 55:521–526
55. Carvalho EB, Curtis WR (1998) Characterization of fluid-flow resistance in root cultures with a convective flow tubular bioreactor. Biotechnol Bioeng 60:375–384

56. Bordonaro JL, Curtis WR (2000) Inhibitory role of root hairs on transport with in root cultures bioreactors. Biotechnol Bioeng 70:176–186
57. Shiao TL, Doran PM (2000) Root hairiness: effect on fluid flow and oxygen transfer in hairy root cultures. J Biotechnol 83:199–210
58. Sanchez Perez JA, Rodrıguez Porcel EM, Casas L´opez JL, Fernandez Sevilla JM, Chisti Y (2006) Shear rate in stirred tank and bubble column bioreactors. Chem Eng J 124:1–5
59. Frahm B, Helmut B, Uwe L (2009) Improving bioreactor cultivation conditions for sensitive cell lines by dynamic membrane aeration. Cytotechnol 59:17–30
60. Gupta DS, Ibaraki Y (2006) Plant tissue culture engineering, focus on biotechnology. Springer, Dordrecht
61. Ramakrishnan D, Curtis WR (1994) Studies in plant science. Advances in plant biotechnology: production of secondary metabolites. In: Ryu DDY, Furusaki S (eds) Fluid dynamic studies on plant root cultures for application to bioreactor design. Elsevier, Amsterdam, pp 281–305
62. Weathers PJ, Wyslouzil BE, Wobbe KK, Kim YJ, Yigit E (1999) The biological response of hairy roots to O-2 levels in bioreactors. In Vitro Cell Dev-Plant 35:286–289
63. Williams GRC, Doran PM (1999) Investigation of liquid-solid hydrodynamic boundary layers and oxygen requirements in hairy root cultures. Biotechnol Bioeng 64:729–740
64. Suresh B, Rajasekaran T, Sathuluri RR, Ravishankar G (2001) Studies on osmolarity, conductivity and mass transfer for selection of a bioreactor for *Tagetes patula* L. hairy roots. Process Biochem 36:987–993
65. Yu S, Doran PM (1994) Oxygen requirements and mass transfer in hairy-root culture. Biotechnol Bioeng 44:880–887
66. Mairet F, Villon P, Boitel-Conti M, Shakourzadeh K (2010) Modeling and optimization of hairy root growth in fed-batch process. Biotechnol Prog 26:847–856
67. Liu R, Sun W, Liu CZ (2011) Computational fluid dynamics modeling of mass transfer behavior in a bioreactor for hairy root culture. Model development and experimental validation. Biotechnol Prog 27:1661–1671
68. Palavalli RR, Srivastava S, Srivastava AK (2012) Development of a mathematical model for growth and oxygen transfer in in vitro plant hairy root cultivations. Appl Biochem Biotechnol 167:1831–1844
69. Prakash O, Mehrotra S, Krishna A, Mishra BN (2010) A neural network approach for the prediction of in vitro culture parameters for maximum biomass yields in hairy root cultures. J Theor Boil 265:570–585
70. Mehrotra S, Prakash O, Khan F, Kukreja AK (2013) Efficiency of neural network-based combinatorial model predicting optimal culture conditions for maximum biomass yields in hairy root cultures. Plant Cell Rep 32:309–317
71. Bhadra R, Morgan JA, Shanks JV (1998) Transient studies of light-adapted cultures of hairy roots of Catharanthus roseus: growth and indole alkaloid accumulation. Biotechnol Bioeng 60 (6):670–678
72. Wang Y, Zhang H, Zhao B, Yuan X (2001) Improved growth of *A. annua* L hairy roots and artimisinin production under red light conditions. Biotech Lett 23:1971–1973
73. Liu C, Guo C, Wang Y, Ouyang F (2002) Effect of light irradiation on hairy root growth and artimisinin biosynthesis of *A. annua* L. Process Biochem 38:581–585
74. Guo C, Liu CZ, Ye HC, Li GF (2004) Effect of temperature on growth and artemisinin biosynthesis in hairy root cultures of Artemisia annua. Acta Botanica Boreali-Occidentalia Sinica 24(10):1828–1831
75. Yu KW, Murthy HN, Hahn EJ, Paek KY (2005) Ginsenoside production by hairy root cultures of *P. ginseng*: influence of temperature and light quality. Biochem Eng J 23:53–56
76. Clemens P (2009) Design principles of photo-bioreactors for cultivation of microalgae. Eng Life Sci 9:165–177
77. Mishra BN, Ranjan R (2008) Growth of hairy-root cultures in various bioreactors for the production of secondary metabolites. Biotechnol Appl Biochem 49:1–10

78. Hilton MG, Rhodes MJC (1990) Growth and hyoscyamine production by hairy root cultures of *Datura stramonium* in a modified stirred tank reactor. Appl Microbiol Biotechnol 33:132–138
79. Hilton MG, Wilson PDG, Robins RJ, Rhodes MJC (1988) In: Robins RJ, Rhodes MJC (eds) Manipulating secondary metabolism in culture. Transformed root cultures – fermentation aspects. Cambridge University Press, Cambridge, pp 239–245
80. Kondo O, Honda H, Taya M, Kobayashi T (1989) Comparison of growth properties of carrot hairy root in various bioreactors. Appl Microbiol Biotechnol 32:291–294
81. Shimomura K, Sudo H, Saga H, Kamada H (1991) Shikonin production and secretion by hairy root cultures of *Lithospermum erythrorhizon*. Plant Cell Rep 10:282–285
82. Caspeta L, Quintero R, Villarreal ML (2005) Novel airlift reactor fitting for hairy root cultures: developmental and performance studies. Biotechnol Prog 21(3):735–740
83. Buitelaar R, Langenhoff AAM, Heidstra R, Tramper J (1991) Growth and thiophene production by hairy root cultures of *Tagetes patula* in various two-liquid-phase bioreactors. Enzy Microb Technol 13:487–494
84. Sim SJ, Chang HN (1993) Increased shikonin production by hairy roots of *Lithospermum erythrorhizon* in two phase bubble column reactor. Biotechnol Lett 15:145–150
85. Suresh B, Thimmaraju R, Bhagyalakshmi N, Ravishankar GA (2004) Polyamine and methyl jasmonate-influenced enhancement of betalaine production in hairy root cultures of *Beta vulgaris* grown in a bubble column reactor and studies on efflux of pigments. Process Biochem 39:2091–2096
86. Kino-Oka M, Hitaka Y, Taya M, Tone S (1999) High density culture of red beet hairy roots by considering medium flow conditions in a bioreactor. Chem Eng Sci 54:3179–3186
87. Ravishankar GA, Venkataraman LV (1990) Food applications of plant cell cultures. Curr Sci 59:914–920
88. Cuello JL, Walker PN, Curtis WR (2003) Design of ebb-and-flow biorector (EFBR) for immobilized hairy root cultures, Part I: preliminary design models and culture parameters. Trans ASAE 46:1457–1468
89. Cuello JL, Walker PN, Curtis WR (2003) Design of ebb-and-flow biorector (EFBR) for immobilized hairy root cultures, Part II: growth studies and model verifications. Trans ASAE 46:1469–1476
90. Watt MP (2012) The status of temporary immersion system (TIS) technology for plant micropropagation. Afr J Biotechnol 11:14025–14035
91. Pavlov A, Bley T (2006) Betalains biosynthesis by Beta vulgaris L. hairy root culture in a temporary immersion cultivation system. Process Biochem 41:848–852
92. Flores HE, Curtis WR (1992) Approaches to understanding and manipulating the biosynthetic potential of plant roots. Ann N Y Acad Sci 665:188–209
93. Wilson PDG (1997) In: Doran PM, Doran PM (eds) Hairy roots: culture and applications. The pilot-scale cultivation of transformed roots. Harwood Academic, Amsterdam, pp 179–190
94. Ramakrishnan D, Curtis WR (2004) Trickle-bed root culture bioreactor design and scale-up: growth, fluid-dynamics, and oxygen mass transfer. Biotechnol Bioeng 88:248–260
95. Weathers PJ, Giles KJ (1988) Regeneration of plants using nutrient mist culture. In Vitro Cell Dev Biol 24:727–732
96. Weathers P, Wyslouzil B, Whipple M (1997) Hairy roots: culture and applications. In: Doran P (ed) Laboratory-scale studies of nutrient mist reactors for culturing hairy roots. Harwood Academic Publishers, Amsterdam, pp 191–200
97. Wyslouzil BE, Whipple M, Chatterjee C, Walcerz DB, Weathers PJ, Hart DP (1997) Mist deposition onto hairy root cultures: aerosol modeling and experiments. Biotechnol Prog 13:185–194
98. Towler MJ, Wyslouzil BE, Weathers PJ (2007) Using an aerosol deposition model to increase hairy root growth in a mist reactor. Biotechnol Bioeng 96:881–891
99. Ranjan R, Ahmed N, Rajesh K, Mishra BN (2009) Design of an ON/OFF mist duty cycle in mist bioreactors for the growth of hairy roots. Biotechnol Bioprocess Eng 14:38–45

100. Ranjan R, Khanna R, Mishra BN (2009) Sustained operation of nutrient mist to grow hairy roots. Asia-Pac J Chem Eng. doi:10.1002/apj.397
101. DiIorio AA, Cheetham RD, Weathers PJ (1992) Growth of transformed roots in a nutrient mist bioreactor: reactor performance and evaluation. Appl Microbiol Biotechnol 37:457–462
102. DiIorio AA, Cheetham RD, Weathers PJ (1992) Carbon dioxide improves the growth of hairy roots cultured on solid medium and in nutrient mists. Appl Microbiol Biotechnol 37:463–467
103. Liu CZ, Wang YC, Zhao B, Guo C, Ouyang F, Ye HC, Li GF (1999) Development of a nutrient mist bioreactor for growth of hairy roots. In Vitro Cell Dev Biol Plant 35:271–274
104. Woo SH, Park JM, Yang J (1996) Root culture using a mist culture system and estimation of scale-up feasibility. J Chem Tech Biotechnol 66:355–362
105. Chatterjee C, Correll MJ, Weathers PJ, Wyslouzil BE, Walcerz DB (1997) Simplified acoustic window mist bioreactor. Biotechnol Tech 11:155–158
106. Williams GRC, Doran PM (2000) Hairy root culture in a liquid-dispersed bioreactor: characterization of spatial heterogeneity. Biotechnol Prog 16:391–401
107. Ramakrishnan D, Curtis WR (1994) Fluid dynamic studies on plant root cultures for application to bioreactor design. In: Ryu DDY, Furusaki S (eds) Studies in plant science. 4. Advances in plant biotechnology: production of secondary metabolites. Elsevier, Amsterdam, pp 281–305
108. Kim YJ, Wyslouzil BE, Weathers PJ (2003) Growth dynamics of *Artemisia annua* hairy roots in three culture systems. Biotechnol Bioeng 83:428–443
109. Albiol J, Campmajo C, Casas C, Poch M (1995) Biomass estimation in plant cell cultures: a neural network approach. Biotechnol Prog 11:88–92
110. Suresh B, Rajasekaran T, Rao SR, Raghavarao KSMS, Ravishankar GA (2001) Studies on osmolarity, conductivity and mass transfer for selection of a bioreactor for *Tagetes patula* L. hairy roots. Process Biochem 36:987–993
111. Srivastava A, Volesky B (1991) Online fluorescence measurements in assessing culture metabolic activities. Appl Microbiol Biotechnol 34:450–457
112. Taya M, Hegglin M, Prenosil JE, Bourne JR (1989) Online monitoring of cell growth in plant tissue cultures by conductometry. Enzyme Microb Technol 11:170–176