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Hairy Root Culture: Bioreactor Design and Process Intensification

Amanda R. Stiles and Chun-Zhao Liu

Abstract The cultivation of hairy roots for the production of secondary metabolites offers numerous advantages; hairy roots have a fast growth rate, are genetically stable, and are relatively simple to maintain in phytohormone free media. Hairy roots provide a continuous source of secondary metabolites, and are useful for the production of chemicals for pharmaceuticals, cosmetics, and food additives. In order for hairy roots to be utilized on a commercial scale, it is necessary to scale-up their production. Over the last several decades, significant research has been conducted on the cultivation of hairy roots in various types of bioreactor systems. In this review, we discuss the advantages and disadvantages of various bioreactor systems, the major factors related to large-scale bioreactor cultures, process intensification technologies and overview the mathematical models and computer-aided methods that have been utilized for bioreactor design and development.

Keywords Bioreactor · Computational fluid dynamics · Hairy roots · Process intensification

Contents

A. R. Stiles · C.-Z. Liu (✉)
National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, People's Republic of China
e-mail: czliu@home.ipe.ac.cn

1 Introduction

Medicinal plants have been used worldwide for thousands of years. Even today, 80 percent of the world's population depends on plant derived medicines for their daily health [1]. Thousands of plant species are used for treatments and therapies. Plant secondary metabolites, which include phenolic compounds, alkaloids, flavonoids, and polysaccharides among others, are the main components for disease treatment. In recent years, increasing numbers of medicinal plants have been discovered for treating infections [2, 3], cardiovascular ailments [4, 5], cancers [6, 7], and even AIDS [8]. In addition, transgenic plants have the potential to produce pharmaceutical proteins, which would have a significant impact on the pharmaceutical industry [9, 10].

However, many medicinal plants are difficult to cultivate and must be collected from the wild, which puts them at risk of becoming threatened or endangered [11]. *In vitro* cultivation offers an efficient method for the large-scale production of plants for medicinal purposes, thereby helping to safeguard critically endangered species. Various in vitro methods are utilized for controllable plant sources for the commercial production of secondary metabolites, including cell culture, callus cultures, organogenesis, embryogenesis and root cultures. The large-scale production of cell, tissue, and organ culture using bioreactors has been accomplished in many species and shows potential for industrial production of naturally occurring as well as novel metabolites.

The cultivation of hairy roots is an in vitro method that has many advantages for commercial production. Hairy roots are developed by infecting explants with *Agrobacterium rhizogenes,* a soil bacterium that integrates a DNA segment into the host plant genome which results in the active proliferation of the roots [12]. Compared to normal root cultures, hairy roots are fast-growing, genetically and biochemically stable, easy to maintain, and are able to grow in phytohormone free media. They offer a continuous source of secondary metabolites, and they are garnering increasing interest for the production of chemicals for pharmaceuticals, cosmetics, and food additives. They are especially useful for the production of secondary metabolites as many products are synthesized in roots but not formed in suspension or callus culture [13]. In addition, due to their stable nature, hairy roots serve as an excellent model system to study plant metabolism and physiology [14]. In addition, they also serve as a promising transgenic system; transformed roots of

many plant species have been widely studied for the in vitro production of secondary metabolites [15–18]. Genetic manipulation can increase the biosynthetic capacity [19, 20], produce multiple secondary compounds, and serve as a heterologous system for compound production, including foreign proteins [21]. Once a transgenic line is produced, a single hair root from the explant tissue serves as a clone [22], and once generated, they are capable of unlimited propagation [23]. Although the majority of research on hairy roots has been conducted in shake flasks [24], production in bioreactors allows for controlled conditions that minimize variations in the yield and quality of the product, and bioreactors allow for the optimization of conditions for increased cell growth and secondary metabolite production [25]. Optimization of bioreactor systems for the cultivation of hairy roots is necessary for their scale-up production for industrial applications.

The cultivation of hairy roots in a bioreactor system creates unique issues, hairy roots are morphologically different among plant species; characteristics such as the thickness, length, hairiness and branching of the roots are affected by both the plant species and the *Agrobacterium* strain used for the induction of hairy roots [25]. In addition, both cell growth and metabolite production are non-homogeneous in hairy roots, further complicating bioreactor optimization [21]. In this review, we discuss the advantages and disadvantages of various bioreactor systems, discuss the major factors related to large-scale bioreactor cultures, discuss process intensification technologies that have been applied in hairy root bioreactors for industrial production, and overview the mathematical models and computer-aided methods that have been utilized for bioreactor design and development.

2 Bioreactor Types

Bioreactors are generally self-contained, sterile environments that include liquid nutrients, inflow and outflow systems for liquid and air, and are designed to optimize and monitor culture conditions. In general, they provide the ability to control micro-environmental conditions, such as pH, dissolved gases, aeration, and temperature [26]. Bioreactors used to culture hairy roots can be broadly divided into either liquid-phase or gas-phase reactors. Briefly, in liquid-phase reactors, roots are submerged in the medium; therefore, they are also sometimes termed ‘submerged reactors’ while in gas-phase reactors, the roots are exposed to air or other gas mixtures, and nutrients are generally delivered to the roots as droplets of various sizes [24]. Additional types include hybrid reactors, which are a combination of liquid and gas-phase reactors, and disposable reactors. Schematic diagrams of several of the different types of hairy root bioreactors are included in a review by Mishra and Ranjan [13]. There are advantages and disadvantages to the many types of bioreactors that have been successfully developed for cultivating hairy roots; therefore, there are many factors involved in selecting the best bioreactor design.An overview of the different bioreactor types is included in Table 1.

Table 1 Advantages and disadvantages of major types of bioreactors

Bioreactor types		Advantages	Disadvantages
Liquid-phase reactors	Stirred tank	Mixing and breaking up air bubbles prevent cell aggregation and enhance oxygenation	High shear force, complicated configuration, increased exposure to contaminants, high energy consumption, difficult to optimize multiple variables
	Airlift and Bubble column	Low shear stress, simple design and construction, low contamination, low maintenance	Foaming induced by large air volumes, non-uniform growth of roots within the reactor (roots can 'float' to the top)
Gas-phase reactors	Nutrient mist	Abundant oxygen supply, low sugar concentration and high space utilization	Complex construction, high energy consumption, labor intensive set-up
	Trickle-bed	Abundant oxygen supply and low energy consumption	May produce a viscous liquid film on the roots creating a high mass transfer barrier, labor intensive set-up

2.1 Liquid-Phase Reactors

In liquid-phase reactors, the culture space is filled with liquid medium and various methods are employed to provide aeration. Because the roots are submerged in liquid medium, mixing and mass transfer become the main bottlenecks in the design and scale-up. Due to the low solubility of gases in the liquid phase, gas exchange limitations and insufficient nutrient transport have been reported [25]. A study by Curtis [27] suggested that at lower tissue concentrations (<10 g/L DW), hairy roots can be grown in virtually any type of liquid-phase reactor, but at higher concentrations (>10 g/L DW), they are likely to encounter scale-up limitations [27]. Design considerations include mechanisms to provide sufficient aeration, nutrient mixing, and immobilization of the roots. The differences in mixing and aeration represent the main design differences among the various types of liquid-phase reactors and several of the most common designs are described below, including stirred tank and pneumatic reactors. Methods for immobilizing hairy roots include meshes (horizontal or vertical), cages, and polyurethane foam [25].

Early studies examined the use of stirred tank reactors, a design commonly used with cell suspension cultures, for the cultivation of hairy roots, including *Calystegia sepium* and *Atropa belladonna* hairy roots for the production of tropane alkaloids [28]. However, the use of the impeller in the traditional stirred tank reactors damaged the sensitive plant tissues, resulting in callus formation and poor biomass production [29]. Therefore, alternate methods to improve the supply of oxygen were tested, and several studies focused on determining ways to improve impeller performance by modifying the internal reactor [30–32]. Stirred tank reactors demonstrated varied success, *Catharanthus trichophyllus* cultivated in both shake flasks and a stirred tank reactor showed a similar alkaloid composition [33], while *Panax ginseng* hairy roots cultivated in a stirred bioreactor, achieved

growth approximately threefold higher than in shake flasks [34]. More recent studies have examined the use of modified stirred tank reactors. Srivastava and Srivastava et al. [35], developed a stirred tank reactor in which the roots were anchored onto a polyurethane foam disk with a low shear impeller for mixing and nutrient transfer placed below the disk. This method allowed for sufficient mixing while protecting the roots from shear stress, although *Azadirachta indica* hairy roots were still unable to produce the same levels of biomass or azadirachtin concentrations obtained via shake flask cultivation [36].

Pneumatic bioreactors, which include both bubble column and airlift reactors, consist of a cylinder with a sparger at the bottom that releases an air or a gas mixture through the solution for both aeration and mixing. Bubble column bioreactors are one of the simplest and easiest types to scale-up. The use of bubbles minimizes shear stress on the cultures, however, the bubbling must be increased as the hairy root biomass increases [37]. Bubble column reactors require low capital, have low operational costs, and contain no moving mechanical parts [21]. However, due to the undefined flow pattern, the liquid is not uniformly mixed [37], and in high density cultures, bubble column reactors often result in reduced growth performance [38]. In high biomass cultures, the bubbles may coalesce, reducing the gas–liquid interface area [21]. The introduction of multiple spargers to deliver oxygen into areas with a high cell density may improve biomass; gas introduced at multiple sections in a multi-compartment bubble column reactor resulted in a high biomass density (in some sections up to 17 g/L), but still provided poor bulk mixing [38]. The use of a microporous polypropylene membrane tubing, in addition to the sparger (required for bulk mixing),to provide supplementary oxygen directly to the root bed, resulted in a biomass increase of 32 % compared to the use of the sparger alone [39]. Various operating parameters may be optimized to improve the biomass and secondary metabolite production in bubble column reactors; for example, the production of alkaloids *by S. parviflora* hairy roots in a bubble column reactor was increased by optimizing the culture period, initial inoculum density, and aeration rate [40].

In contrast to bubble column reactors, airlift bioreactors contain a draft tube (either internal or external) to prevent bubbles from coalescing, and to enhance oxygen mass transfer by increasing the number of bubbles. Airlift reactors reduce shear stress, distribute shear stress more evenly, consume little energy, and promote a cylindrical mixing of the medium. Airlift bioreactors have been used extensively used for hairy roots since the initiation of hairy root bioreactor studies, for species including *Panax ginseng* [41], *Armoracia rusticana* [42], *Trigonella foenumgraceum* [43], *Lippia dulcis* [44], *Lithospermum erythrorhizon* [45], *Ophiorrhiza pumila* [46], and *Echinacea purpurea* [47]. In some cases, airlift bioreactors have been highly effective compared to shake flask cultures; *Pueraria phaseoloides* hairy roots cultivated in a 2.5 L airlift bioreactor produced 200 times as much puerarin as in a 250 ml flask culture [48]. The growth of *Panex ginseng* hairy roots inoculated into a 5 L airlift bioreactor increased by approximately 55-fold after 39 days in a 5 L airlift bioreactor, and 38-fold after 40 days in a 19 L airlift bioreactor [34]. In airlift reactors, both the liquid and the aeration are driven

by externally supplied air; however, similar to bubble column reactors, they are generally not suitable for high-density cultures because they result in insufficient mixing and oxygen mass transfer [37].

2.2 Gas-Phase Reactors

In gas-phase reactors, the roots are exposed to either an air or a gas mixture and the nutrients are delivered as droplets of different sizes. They have been widely utilized in plant tissue culture, and are useful for hairy root cultures because they provide an abundant oxygen supply. In gas-phase reactors, the deposition of nutrients is the key step in nutrient mass transfer; however while some deposition is required, excessive deposition will result in a thick liquid layer on the root surface that inhibits gas transfer [49]. In addition, if drainage is insufficient in gas-phase reactors, the root bed may retain liquid and further inhibit gas transfer [50]. In contrast to liquid-phase reactors, in which the root hairs increase the flow resistance of the media, in gas-phase reactors, the root hairs are believed to be beneficial for growth because they enhance mist capture and may improve reactor performance [51]. Although gas-phase reactors result in improved oxygen transfer, they still require a matrix for anchoring the hairy roots (such as mesh trays, stainless steel mesh cylinders), and the requirement for uniform loading may be labor intensive [25]. Examples of gas-phase reactors include: nutrient mist, nutrient sprinkle, and trickle bed reactors.

In mist bioreactors, the nutrient mist may be produced by ultrasonic methods, nozzles, or compressed air. The mist dispersed in the bioreactor has a large specific surface area that leads to a high oxygen transfer rate from gas into medium, and may account for the higher biomass production in mist bioreactors compared with other types. Compared with both shake flasks and bubble column reactors, mist reactors are less oxygen-limited; this was demonstrated by an analysis of the expression of alcohol dehydrogenase, an indicator of oxygen stress, in hairy root cultures. In a mist bioreactor there was no detectable expression up to a packing density of approximately 37 % (v/v) while in contrast, there was a significant expression of alcohol dehydrogenase in roots grown in bubble column bioreactors at densities of only 6 % [52]. Parameters that may be adjusted in a mist reactor to optimize nutrient delivery include the misting cycle time and the medium flow rate. A mist bioreactor scaled up from 1–20 L for *A. hypogaea* hairy root cultures demonstrated that increasing the misting cycles longer that 2–3 min was detrimental, but increasing the medium flow rate during the exponential growth phase increased the growth rates and biomass yields [53].

Nutrient sprinkle reactors produce larger droplets than mist reactors. Kochen et al. [54] developed and tested a nutrient sprinkle reactor for the production of ginsenosodies from *Panax quinquifolium*; although the biomass increase in the nutrient sprinkle reactor was slightly lower than shake flask cultures, the ginsenoside content was doubled when the cultivation of both *Salvia officinalis* hairy

roots and shoot cultures was examined in a nutrient sprinkle bioreactor; the hairy roots achieved an 18-fold increase in biomass after 40 days of culture and the production of rosmarinic acid was approximately 1.3-fold higher in the hairy roots compared to shoot cultures. Overall, the reactor was considered unsuitable for the cultivation of shoots due to hyperhydricity issues [55]. *Salvia sclarea* hairy root cultures cultured in a nutrient sprinkle reactor produced 9 and 3.8 times as much aethiopinone and salvipisone, respectively, as roots cultured in shake flasks [56].

Trickle-bed bioreactors produce the largest sized droplets compared with mist and nutrient sprinkle bioreactors; this results in the formation of a thicker liquid film on the plant tissue surface and creates a disadvantage for gas transfer [24]. In addition, the tendency for the root bed to accumulate liquid and the absence of agitation are major limitations in the large-scale design of trickle-bed bioreactors [57]. However, they supply a larger volume of liquid nutrients to the cultures and use less energy compared with mist bioreactors. Ramakrishnan et al. [58] scaled-up a 14 L trickle-bed bioreactor for *Hyoscyamus muticus* hairy root culture, and their analysis of the mass transfer and fluid dynamic characteristics provided a method for the design in the scale-up of trickle-bed bioreactors [58].

Several comparison studies have been conducted to determine the optimal bioreactor type for a particular species of hairy roots. The production of *Artemisia annua* hairy roots was compared in mist and bubble bioreactors, and while the artemisinin content of *Artemisia annua* hairy roots was higher in the mist reactor [59], the overall biomass was higher in the bubble column reactor [60]. A comparison of the growth and productivity of transgenic tobacco hairy roots in a mist bioreactor and an airlift bioreactor showed that the synthesis of murine interleukin 12 was increased by approximately 50 % in the mist bioreactor [9]. *Tagetes patula* hairy root cultures cultivated in a bubble bioreactor, a sprinkle bioreactor, and a mist bioreactor achieved the highest growth rate, biomass production, and metabolite production in the mist bioreactor [61]. Srivastava and Srivastava [36] examined azadirachtin production by *Azadirachta indica* hairy roots in a stirred tank reactor, bubble column reactor, nutrient spray reactor, and nutrient mist reactor and found that the nutrient mist reactor was able to produce biomass and azadirachtin levels most similar to those achieved in optimized shake flask cultures [35].

2.3 Novel Bioreactors

Although overall, gas-phase reactors have the advantage of improved oxygen transfer efficiency, a disadvantage of gas-phase reactors is the necessity for the manual distribution of the roots in the growth chamber, a labor intensive process. One method to address this issue was the development of a hybrid reactor, a reactor in which the initial phase of the reactor was liquid-phase to allow the roots to attach uniformly to the anchoring system, and once attached, the reactor was switched to a gas-phase reactor. A large-scale (400L) reactor was developed in the late 90's using this method (a combination of a bubble column and spray reactor)

for *Datura stramonium* hairy roots [62], and Ramakrishnan et al. [50] also developed a hybrid reactor in which the reactor was initially run as a bubble column, and then switched to a gas-phase reactor for the improved efficiency.

Disposable bioreactors are another alternative to the traditional culture systems. Disposable reactors utilize sterile plastic chambers that may be inoculated and then discarded once the roots are harvested, thus lowering operation costs by eliminating the need for cleaning or sterilization [63]. *Hyoscyamus muticus* and *Panax ginseng* hairy roots were cultivated in wave bioreactors, disposable bioreactors consisting of screw-cap sealed plastic bags under laminar fluid flow conditions. Under optimal culture conditions, the *Hyoscyamus muticus* root biomass increased by approximately 120-fold after 28 days, and the *Panax ginseng* biomass increased by 28-fold (compared to only 12.1-fold in a spray reactor) [63].

3 Bioreactor Parameters

Secondary metabolite biosynthesis in hairy roots is genetically controlled, but is influenced by nutritional and environmental factors. In addition, the rheological properties of hairy root cultures vary from one species to another and sometimes even within clones of the same species [13]. Multiple factors must be considered for the successful scale-up of bioreactor systems, including the physiology, morphology, and stress sensitivity of hairy roots. Submerged hairy roots tend to form dense clumps due to their tendency to form lateral branches and the hairiness of the roots [64, 65]. In liquid-phase reactors, these dense clumps resist fluid flow and therefore the delivery of nutrients to the tissues, creating one of the major challenges in reactor scale-up.The main bottleneck in scale-up, is the delivery of sufficient oxygen, but other factors, such as light, other gases, temperature, nutrients, and inoculum conditions also affect the growth and productivity of hairy root cultures [37].

3.1 Oxygen and Other Gases

Dissolved oxygen plays an important role in the bioreactor microenvironment, though oxygen requirements may vary from species to species. Hairy roots cultured in bioreactors have a tendency to form dense root clumps that limit oxygen transfer; therefore, understanding oxygen transfer and supply has been one of the most highly studied research topics in hairy root bioreactor design. In general, it is essential that the dissolved oxygen concentration remains above a critical level at all the times for optimal cell growth [66]. Shiao and Doran found that as root hairiness increased, the oxygen mass transfer boundary layer increased, limiting oxygen consumption in hairy root cultures [67]. A mathematical model developed to estimate the oxygen mass transfer coefficient in bubble column reactors

demonstrated that due to the low solubility of oxygen and the high oxygen consumption of hairy roots, the oxygen concentration near hairy roots was significantly different from the bulk fluid [68]. When dissolved oxygen drops below a certain point, respiration is impaired, fermentation begins, and toxic byproducts are produced.

In liquid-phase reactors, the oxygen concentration is generally measured using a sterile electrode, and can be regulated by agitation, aeration, gas flow, and bubble size. Analyzing the transport of oxygen requires both characterizing the oxygen gradients and the thermodynamic equilibria that determine the solubility of oxygen in the media and plant tissue [69]. The oxygen concentrations in liquid cultures depend on the presence of oxygen in the gas phase above the medium, in the air bubbles inside the medium, and on the dissolved oxygen in the medium itself. Oxygen transport in hairy root cultures in a liquid-phase reactor is mainly influenced by the mass transfer resistance at the liquid–solid phase, rather than gas–liquid transfer [23]. Early studies using high liquid velocities to remove the hydrodynamic boundary layers, demonstrated that culture growth did not continue exponentially and indicated that the mucilage and root hairs are a barrier to oxygen transfer [57]. Other studies corroborated that the root hairs contributed to a pressure drop in convective flow reactors [70, 71]. Overall, due to the morphology of hairy roots, it is unlikely that it will be possible to completely eradicate oxygen limitations in liquid-phase reactors. It is possible to optimize and improve growth, using methods such as increasing the aeration rate; increasing the aeration rate from 0.002 m^3/h to 0.012 m^3/h improved cichoric acid production of *Echinacea purpurea* cultures cultivated in a modified air lift reactor [47].

Gas-phase reactors have the advantage on greater oxygen transfer and reduce the accumulation of mucilage; however, the thick roots can trap liquid, thereby reducing the contact of the roots with the gas phase. Williams and Doran [57] have suggested this could be overcome with the use of mechanical or pneumatic agitation [57], and methods to reduce cycle on time and study drainage characteristics have been tested to reduce this issue. For example, Ramakrishnan et al. found that respiration in hairy roots increased sharply with increased liquid spraying rate at relatively high tissue concentration in trickle-bed reactor [50].

Many methods have been tested to improve oxygen availability for hairy roots in bioreactors. In liquid-phase reactors, additional tubing has been added to improve oxygen availability, such as Kanokwaree et al. added microporous polypropylene tubing to bubble bioreactors containing *Atropa belladonna* hairy root cultures, thereby diminishing the oxygen limitation and improving both biomass and the accumulation of metabolites [39]. Kino-Oka et al. [72] found that increasing the medium flow rate in a bubble column reactor enhanced the dissolved oxygen concentration but simultaneously increased shear stress. To overcome this, a radial flow reactor was constructed that successfully released the oxygen transfer barrier in high-density red beet hairy root cultures [72]. A transgenic method was also tested to enhance growth under low oxygen conditions; transgenic *Arabidopsis thaliana* L hairy root lines that contained alcohol dehydrogenase and pyruvate decarboxylase were generated. The transformed root lines

showed a similar growth rate under low oxygen conditions as those under full aeration [73].

The biomass and secondary metabolite production is affected by the gas composition in the media. The gas composition within a bioreactor is impacted by its volume, the volume of the media, and the bioreactor design. In addition to the oxygen concentrations within a bioreactor, carbon dioxide and ethylene also play a role. Carbon dioxide has the potential, in some species, to improve root growth. For example, beet hairy roots cultured in a nutrient mist reactor enriched with 1 % carbon dioxide showed a 15 % increase in biomass compared with tissue cultured in ambient air [74]. This response was due to the fact that it reduced the lag phase of the culture, allowing the culture to accumulate biomass faster, not because it increased the actual growth rate. However, the effect of carbon dioxide may vary dependent on the species; *A. annua* hairy roots grown in enriched carbon dioxide appeared healthier, but did not grow faster [75]. Sung and Huang [76] examined the effect of ethylene on the growth and secondary metabolite production of *Stizolobium hassjoo* hairy roots. The accumulation of ethylene in the headspace resulted in a reduction in both biomass and secondary metabolite production (L-DOPA), and when an ethylene inhibitor was added, both biomass and L-DOPA increased [76].

3.2 Light and Temperature

Light plays a role in both growth and secondary metabolite production, and due to the morphology of the hairy root cultures, it is difficult to achieve uniform illumination. The stimulatory effect of light on the formation of secondary compounds has been demonstrated in plant species including *Perilla fruitescnens* and *Artemisia annua* [77–79], and *Ipomea aquatica* hairy roots grown in the light produced twice the biomass and four times the peroxidase as roots grown in the dark [80]. Many hairy root lines have been documented to change colors, to colors such as green or purple when exposed to light [78, 82]. In general, the green color is due to the development of chloroplasts that are fully capable of photosynthesis and the metabolic capabilities of the green roots are distinct from their non-green counterparts [83]. *Echinacea purpurea* hairy root cultures exposed to light develope a dark purple color which is related to anthocyanin production [77]. Abbasi et al. [77] studied the effect of light on the accumulation of caffeic acid derivatives (CADs) in *Echinacea purpurea* hairy root cultures and found that all of the CADs were significantly different between light-and dark-grown roots. Bhadra et al. [81] studied the effect of light on indole alkaloid accumulation by *Catharanthus roseus* hairy root cultures, and found the alkaloid total yield was also significantly different between light- and dark-grown hairy roots, and that the production of certain alkaloids during specific growth phases shifted when light conditions were altered [81]. Hairy roots that do not change colors in response to light may also show profound alterations in secondary metabolism in response to light [24].

The photoperiod and light intensity are interrelated, and both are important to hairy root cultivation. For example, Liu et al. [84] found that 16/8 h light/dark was optimum for artemisinin production by *Artemisia annua* hairy root cultures, and Wang et al. [78] found that red light had the greatest impact compared to all other light regimes. A comparison of *Panax ginseng* hairy root cultures under several different light regimes (dark, fluorescent, metal halide, monochromatic red, monochromatic blue, and red plus blue) showed that the ginsenoside production was highest in cultures grown under fluorescent light, while the biomass was higher in cultures grown under either red light or in the dark [85]. Temperature also plays an important role and may vary from species to species. A study on the effects of temperature on *Solanum aviculare* hairy roots identified 25 °C as the optimal temperature [86], while Hilton and Rhodes [87] found that *Datura stramonium* hairy roots cultivated at 30 °C had a biomass approximately 4-fold greater, and the production of hyoscyamine was up to 7-fold higher. In *Panax ginseng* hairy root cultures, a comparison of different temperatures demonstrated that the biomass of the hairy roots was highest in cultures incubated at 20 °C/13 °C for 16/8 h day cycles, while the production of total ginsenosides was optimum at cultures incubated at 25 °C/25 °C [85].

3.3 Nutrient Medium Composition

In experiments with *Panax ginseng* hairy roots, Sivakumar et al. [88] found that the commonly utilized MS medium resulted in low hairy root growth, biomass, and ginsenoside content, indicating that the nutrient composition could be optimized. They identified nutrient availability as the major chemical factor for scale-up and suggested that mineral elements are an important regulatory factor in hairy root growth and biomass production [88]. In addition, periodic estimations of specific nutrients at different periods in bioreactors can provide information regarding nutrient uptake, biomass, and metabolic production. Generally, the medium composition is modified with respect to its concentration of carbon, nitrogen, phosphorus [38, 89], and macronutrients [88]. A factorial experiment using *Artemisia annua* hairy roots was employed to determine the relationship between four factors (phosphate, nitrate, sucrose, and culture inoculum age) at three levels; results indicated there was a significant interaction between sucrose and nitrate although the other factors were less interactive [90]. Depending on the relationship between the biosynthesis of the secondary metabolites and biomass production, the media can either optimized for both growth and secondary metabolite production simultaneously or a two-stage strategy may be developed. A study evaluating the production of tropane alkaloids by *Datura stramonium* L. hairy roots used a factorial design and mathematical modeling approach to evaluate the relationship between nutritional status and elicitation [91]. They concluded that simulation with their developed model allowed them to predict the response to elicitation in response to nitrate and calcium levels, and that if hairy

roots were nitrate limited; they showed a lowered response to jasmonic acid elicitation. A study on the effect of MS medium dilution on the biomass and accumulation of phenols and flavonoids in *Hypericum perforatum* adventitious roots found that the peak root growth was at four weeks using half-strength MS, and the content of total phenols and flavonoids were also highest using the quarter or half-strength MS [92].

4 Process Intensification

Process intensification approaches are utilized in plant cell and tissue cultures to enhance biomass production and metabolite yield. Elicitation, the introduction of compounds that trigger an increase in metabolite production, and ultrasound, a method utilized to enhance mass transfer in plant cell cultures, have both been applied to hairy root bioreactor cultures.

4.1 Elicitation

Elicitors are compounds that trigger the increased production of compounds in plant cells such as pigments, flavones, phytoalexins, and other chemicals related to defense [93]. In general, elicitors are classified as either biotic or abiotic. Biotic elicitors are derived from a pathogen or from the plant itself [94], while abiotic elicitors are either physical or chemical compounds. The effectiveness of elicitation depends on multiple variables, such as the specificity of the elicitor, the concentration, treatment interval, culture growth stage, medium composition, and light.

Abiotic elicitors are generally less expensive than biotic, and biotic elicitors also have the disadvantage that they may require additional facilities for the cultivation of the source microorganism [95]. In many studies, both abiotic and biotic elicitors are tested. For example, Pitta-Alvarez et al. [96] tested the effects of different biotic (salicylic acid, yeast extract) and abiotic ($CaCl_2$, $AgNO_3$, $CdCl_2$) elicitors on the accumulation and release of scopolamine and hyoscyamine in *Brigmansia candida* hairy root cultures [96]. Salicylic acid significantly increased the release of both alkaloids, $AgNO_3$ and yeast extract preferentially increased the release of scopolamine, and $CdCl_2$ increased the release of both alkaloids but also inhibited growth. *Azadirachta indica* A. Juss hairy roots exposed to a biotic elicitor (the fungus *Claviceps purpurea*), 100 mM jasmonic acid, or 200 mM salicylic acid achieved an approximate 5, 6, and 9-fold increase in azadirachtin yield, respectively [97]. A review by Georgiev [95] includes additional examples of secondary metabolite yield increases in response to elicitation of hairy roots [95].

Methyl jasmonate is a potent elicitor due to its ability to induce plant signal transduction pathways; *Salvia sclarea* hairy roots grown in a sprinkle bioreactor showed a 9-fold increase in aethiopione and a 3.8-fold increase in salvipisone when they were exposed to 125 μM methyl jasmonate for 7 days [56]. The application of 100 μM methyl jasmonate applied to *Silybum marianum* hairy roots cultured in a 2 L airlift bioreactor, resulted in a 1.6-fold increase in silymarin accumulation after only 72 h [98], and *Artemisia annua* L. hairy roots treated with 150 mg chitosan/L, 200 μM methyl jasmonate, or 2 mg/mL yeast extract resulted in a 6-fold, 3-fold, and 5-fold increase in artemisinin production, respectively [99].

Although elicitors may increase metabolite production, they may also simultaneously reduce biomass. *Beta vulgaris* hairy roots exposed to biotic elicitors including purified microbial glycans (200-500 mg/L), whole microbial culture extract (0.25–1.25 %), and culture filtrates (5–25 %, v/v) achieved significantly increased betalain production in the *Penicilium notatum* DCP-treated cultures, but all elicitors resulted in a reduction of biomass, reducing overall yields [100]. However, this is not always the case; vanadyl sulfate ($VOSO_4$), an abiotic elicitor, resulted in an 8-fold increase in biomass compared to the control in *Ambrosia artemisiifolia* hairy root cultures when 50 mg/L was applied for 72 h to 16-day-old cultures [101]. Due to the fact that in some cases elicitation inhibits root growth, a two-stage culture strategy, in which elicitation is conducted once growth is complete, may be used in scaled-up processes. For example, in elicitation studies with *Beta vulgaris* hairy roots cultured in a bubble-column reactor, the elicitor was added at late exponential phase, resulting in approximately a 47 % increase in betalain production compared to controls [100].

Elicitors may also increase oxidative stress; *Silybum marianum* hairy roots exposed to 100 uM methyl jasmonate for 72 h showed the increased expression of antioxidant enzymes such as ascorbate peroxidase and guanicol peroxidase by 1.3 and 3.2-fold, respectively [98]. Although our current understanding of the mode of action of elicitors is related almost exclusively to secondary metabolism, primary metabolism may also be affected. Vasconsuelo and Boland [94] suggest that elicitation may also affect processes that regulate levels of secondary metabolites, such as vacuolar transport and compound storage, and that increasing our overall understanding these processes will be helpful to improve secondary metabolite production [94].

4.2 Ultrasound

Ultrasound has been widely applied in chemistry, chemical engineering, medicine, and biology for its chemical, physical and biological effects. In plant cell cultures, ultrasonic treatments have been employed to enhance metabolism and mass transfer, examples include, *Lithospermum erythrorhizon*,*Panax ginseng*, and *Taxus chinensis* [102–104]. The effective distance of the ultrasound wave is short due to the attenuation of ultrasound wave [105, 106]; however, ultrasound has been

successfully employed in hairy root cultures to intensify mass transfer in order to stimulate both root growth and secondary metabolite production. Cichoric acid production was increased in *Echinaciea purpurea* hairy root cultures by the use of six minute ultrasound treatments in a 2 L bioreactor [47]. The acoustic model has been utilized to understand the effects of ultrasound treatments on fluid flow [107, 108]; however, an optimal design for the scale-up of a sonobioreactor that would provide uniform ultrasound has not yet been developed.

5 Bioreactor Design and Scale-Up

5.1 Mathematical Models

Root tissues are very different from the common microbial or cell cultures, and bioreactors for hairy root culture are more difficult to operate, control, and scale-up. The high number of variables results in complicated methods of operation. Developing growth models for bioreactor cultures is part of the necessary research for the design, operation, and scale-up for the industrial production of hairy roots. The use of mathematical models aids in the design of reactors to improve biomass, secondary metabolite production, and secondary metabolite recovery.

Several methods have been established to monitor the process of plant cell growth, Kim et al. [109] developed a growth model using *Tagetes erecta* T3 hairy roots as a model system [109]. In this study, a mathematical model of branching dynamics was constructed to simulate the branching rules for hairy roots, and the growth rate and biomass were monitored using the population balance equation and the Monod equation. This model functioned to illustrate the root growing process and aid in bioreactor scale-up. More recently, a medium-throughput automatic image recognition system was developed to provide quantitative data regarding the growth patterns and secondary metabolite production (the red betacyanin pigment) of *B. vulgaris* hairy roots. In this system, they quantified three main growth processes; tip elongation, branching and secondary thickening, to evaluate growth, and quantified the red color produced by betacyanin to evaluate secondary metabolite production. This type of approach helps to provide quantitative data that may be used for the creation of a structured growth model for hairy roots via mathematical modeling [110].

In both shake flasks and bioreactors, hairy roots tend to form a dense network of tissue with younger, more lightly packed roots surrounding the older core. Bastian et al. [111] developed a general growth model that included hairy root elongation, branching, secondary growth, and nutrient transport functions in dense root networks. After certain simplifications, the model was simulated with the experiment data using *Ophiorrhiza mungos* hairy root culture. This model could be used to describe the uptake kinetics and biomass increase of *O. mungos* hairy roots grown in shake flasks, and helped with the optimization of process parameters in hairy

root culture. Oxygen transfer is a critical factor in bioreactor cultures because in high density cultures; the oxygen uptake rate is generally faster than the oxygen mass transfer rate. Early experiments determined that oxygen consumption within the hairy root mass was restricted by the rate of convective mass transfer within the mass, and increasing the flow rate around the mass would induce higher rates of oxygen consumption [112].

The growth and metabolism of plant cells are distinct when they are cultivated in different environments; therefore, in order to achieve maximum production, it is necessary to monitor the cell and tissue growth, nutrient consumption, mass transfer, and rheology. Ramakrishnan and Curtis [58] detailed the oxygen transfer process in a plant tissue culture system using the uptake equilibrium and diffusion equilibrium. The mass transport process was divided into several steps including transport in the gas phase, transfer from gas into liquid phase, and transport from liquid to tissue. They constructed models of oxygen transport and oxygen consumption to estimate the solid–liquid mass transfer coefficient in hairy root cultures in a trickle bed bioreactor, which could then be used for the design of scaled-up reactors [58]. The mathematical analysis of oxygen transport into the plant tissue culture provided a quantitative understanding of the culture conditions; this is helpful for understanding oxygen transport limitations, which could then guide bioreactor design. Recently, a mathematical model was developed to evaluate and optimize operating parameters such as the mist flow rate, the nutrient concentration, the timing for the mist on/off cycle, and drainage characteristics within a nutrient mist reactor [49]. In a similar study, they developed a mathematical model for a nutrient mist reactor to cultivate *A. annua* hairy roots. Intermittent misting helps to ensure that the root surfaces are sufficiently wetted, while short cycles allow for the liquid to drain. The results of this study indicated that intermittent on/off cycles were effective for sustained operation, and to prevent water-logging of the bed, it was necessary that the mist has a short 'on' cycle. They determined that the mist could be switched off for 90 % of the run time in an optimized system [113].

5.2 Computation Fluid Dynamics (CFD)

Models of growth and mass transfer of plant tissue are necessary for bioreactor design and scale-up. However, these models ignore the hydrodynamic environment which also influences the growth and metabolism of hairy roots cultured in bioreactors. Monitoring the hydrodynamics helps to evaluate the fluid characteristics (such as fluid flow, shear stress, and turbulent intensity) that are key parameters in bioreactor design and scale-up. Computational fluid dynamics (CFD) offers a novel approach for investigating hydrodynamic behavior and designing reactor structures. CFD has been used in the simulation of hydrodynamic turbulent shear stress in a plant cell culture bioreactor [114], analysis of hydrodynamics for the design of trickle-bed reactors [115], the optimization of the

inner structure in a flat algal photobioreactor [116, 117], design of the sparger in a bubble column bioreactor [118, 119], design of the impeller in a stirred tank reactor [120], and for the optimization of the height to diameter ratio in bubble column reactors [118]. The fluidization behaviors induced by ultrasound were also simulated with the help of CFD [107, 108].

5.3 CFD for Hairy Roots

Recently, Liu et al. [121] used CFD to investigate the hydrodynamics and mass transfer in an internal-loop airlift bioreactor containing *Echiniacea purpurea* hairy root cultures [122]. In this study, the CFD model was based on a porous media model (representing the hairy roots) and a discrete population balance model (representing bubble number and diameter). A two-dimensional axisymmetric model was used to describe the bioreactor due to its axial symmetric structure. The porosity of the hairy root clump was measured and calculated as follows:

$$\gamma = \frac{V - m/\rho_{root}}{V} \tag{1}$$

where V was the volume of the hairy root clump, m was the mass of the hairy root clump and ρ_{root} was density of the hairy roots of 1 g/ml.

The resistance coefficients were extrapolated after determining the pressure drop through the hairy root clumps by packing the hairy roots into a 2.54 cm × 40 cm glass tube and measuring the water flow rate under defined pressure drops. These values were used to determine the resistance coefficients D and C_2 in Eq. 2.

$$\Delta p = C_2 \frac{1}{2} \rho |\vec{u}|^2 \Delta n + D\mu |\vec{u}| \Delta n \tag{2}$$

where Δn was the porous media thickness (the glass tube height, m), ρ was the density of the fluid, and μ was the water flow rate under the pressure drop change Δp. In a test using *Echiniacea purpurea* hairy roots, parameters including liquid and gas velocity, gas holdup, mass transfer rate, and distribution of oxygen concentration in the airlift bioreactor were simulated using the CFD model. A Euler–Euler multiphase model was used to simulate the hairy root culture process. There are two types of mass transfer in the bioreactor; interphase mass transfer, in which oxygen is transferred from gas phase into liquid phase, and intraphase diffusion, the diffusion of the dissolved oxygen within the liquid phase.

Testing the parameters demonstrated that the porosity of the hairy roots changed during the culture period, from 1 to 0.7, which thereby changed the water flow resistance coefficients. As the fluid flow resistance increased, this influenced the nutrient supply for the roots. As the dissolved oxygen concentration increased, the oxygen consumption rate increased. The modeling results indicated that the

dominant factors in the oxygen mass transfer in the hairy root culture were the liquid flow and turbulence. It also predicted that the concentration of dissolved oxygen would increase from the bottom to the top of the hairy root clump, and this was verified experimentally. The use of CFD provides an elegant method to guide future bioreactor design and scale-up as well as process intensification for large-scale bioreactor culture of hairy roots.

Overall, the experimental data validated the model, and provided a system that could be used to model the dissolved oxygen concentration distribution, which is difficult to determine during the process of cultivating hairy roots in a bioreactor. This model provided a simple and efficient method to predict the dissolved oxygen concentration and consumption rate distribution, and allows researchers to predict and optimize the bioreactor design and scale-up, as well as develop process intensification strategies, without having to perform numerous time-consuming bioreactor experiments.

This same model was subsequently used to simulate hydrodynamics and oxygen mass transfer in an ultrasound-intensified *Echinacea purpurea* hairy root culture [121]. The results of the simulated model, and the experimental data, demonstrated that the ultrasound intensified oxygen mass transfer in the hairy root clump, thereby stimulating growth and cichoric acid biosynthesis.

6 Conclusions

Hairy roots are an efficient system for the production of secondary metabolites, and as the global demand for these economically important compounds rises, there will a heightened interest in their production at commercial-scale. In addition to secondary metabolites, hairy roots are likely to be increasingly utilized for the production of recombinant proteins, especially for medicinal purposes. A method recently developed utilizing the combination of a chimeric super promoter, a translation enhancer sequence, and signal peptide for secretion of the recombinant protein into the media, resulted in transgenic tobacco hairy roots producing reporter protein at levels that were ten-fold higher than produced in tobacco leaves [123]. Hairy roots are also being used in phytoremediation, such as the use of *Tagetes patula* hairy roots for the decolorization of dyes [124], transgenic tobacco hairy roots for the overexpression of peroxidases for phenol removal [125], and *Brassica napus* hairy roots for the removal of 2,4-dichlorophenol from aqueous solutions [126]. Continued research in the scaled-up production of hairy roots in bioreactors will be necessary. Although methods have improved, the scale-up from shake flasks to bioreactor systems still faces many challenges; providing hairy roots with optimal levels of nutrients, oxygen, adjusting operating parameters as the culture matures, and optimizing elicitor type and timing are still being researched. In addition, in contrast to other types of culture systems, hairy roots are often morphologically varied from species to species, or even within a species, therefore optimization may be a very individual process for a specific commercial

product. The continued development and improvement of low-cost and low-maintenance systems will enhance commercialization, as well as develop methods that streamline the harvesting of hairy roots from the bioreactor system. The development over the last decade, and especially in the last few years, of computational methods for the improvement of bioreactor design, such as mathematical modelling and computational fluid dynamics will be invaluable in this endeavour.

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