Current Biotechnology, 2018, *7*, 417-427

REVIEW ARTICLE

Bioreactors for Hairy Roots Culture: A Review

Sadaf Aiman Khan, Mohammed Haris Siddiqui and Khwaja Osama*

Department of Bioengineering, Integral University, Lucknow, India

ARTICLE HISTORY

Received: November 16, 2018
Revised: January 02, 2019
Accepted: January 04, 2019

DOI:
10.2174/2211550108666190114143824

Abstract:

Background: Plants are a source of a variety of secondary metabolites. *Agrobacterium rhizogenes* mediated hairy root cultures offer a great advantage for the production of these metabolites in large amounts in comparison to cell suspension cultures as they have the capability of fast growth along with genetic stability. In order to commercialise secondary metabolite production, the mass production of hairy root cultures is of paramount importance. Various conventional bioreactors, broadly classified as liquid phase and gas phase reactors, have been employed for this purpose which includes airlift, bubble column, stirred tank, trickle bed, nutrient mist, spray reactors and others.

Objective: This review discussed various aspects of hairy root culture in bioreactors.

Method: Peer-reviewed research literature was searched and screened for information about the evolution of designs of bioreactors used for hairy roots culture. Data was gathered after a keen search from research and review articles and is presented in this review paper.

Results: The major limitations of the conventional type bioreactor systems were high shear stress and oxygen deficiency, thus the demand for improved designs led to the evolution and designing of various types of bioreactor systems with low shear stress and better oxygen uptake in order to enhance yield productivity. Amidst these modifications in reactors, hybrid reactors, which are a combination of liquid phase and gas phase reactors, offer a very promising approach for commercialisation of secondary metabolite production using hairy root cultures.

Conclusion: Although many efforts have been done to obtain a bioreactor configuration for highest biomass possible, there are still chances of improvement to get the most suitable bioreactor that could provide high oxygen mass transfer, better growth characteristics, homogenous culture environment and minimum shear stress.

Keywords: Hairy roots, secondary metabolite, stirred tank reactor, airlift reactor, bubble column reactor, nutrient mist reactor.

1. INTRODUCTION

Agrobacterium rhizogenes, the natural genetic engineer, is a soil bacterium responsible for genetic transformation in plants. Since its first use in higher organisms (tobacco plants) in 1973 by C. Ackermann, this bacteria has become a potential tool for genetic engineering in plants. *Agrobacterium rhizogenes* transfers the T-DNA from their Ri plasmid to the chromosomal DNA of the plant cell at the wound site, which induces the formation of hairy root [1, 2]. Over the past few years, hairy roots have been used for a variety of purposes, such as metabolic engineering, recombinant protein production, phytoremediation, plant-plant interaction and they might also be used as a potential system for biofuel production in the future [3]. Hairy root technology potential has also been considered in exploiting new biological insights into a variety of plant species (Fig. **1**). Apart from these potentials, the major use of hairy root system has been in secondary metabolite production [4].

Plants are a valuable source of a variety of secondary metabolites such as steroids, alkaloids, flavonoids, terpenoids, anthocyanins, quinins, and lignans, which are utilised as drugs, flavours, agrochemicals, dyes, fragrances, insecticides, biopesticides, as well as food additives. Amongst the 30,000 (approx.) known natural products, 80% have their origin from plants. Extraction of phytochemicals from plants poses certain challenges. A large agricultural land is required to grow plant biomass for the extraction. *In vitro* culture of plant cells offers a more attractive alternative than growing the whole plant for the production and extraction of secondary metabolites. A number of plant cell suspension cultures have been observed that are able to produce a better amount of high-value secondary metabolites in comparison to parent plants [4]. For large production of plant-derived secondary

*Address correspondence to this author at the Department of Bioengineering, Integral University, Lucknow, India; E-mail: osama@iul.ac.in

2211-551X/18 $58.00+.00

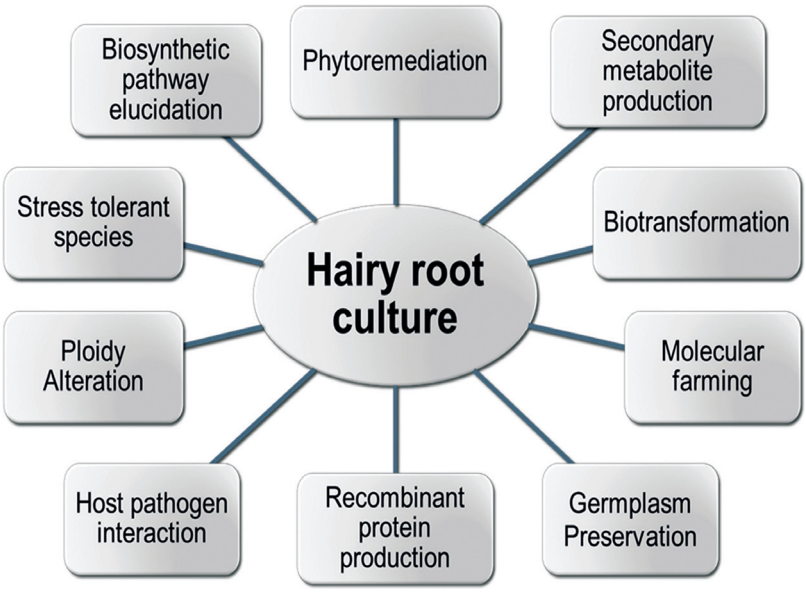

Fig. (1). Various areas of application of hairy roots.

metabolites, the culture of plant cells in industrial size vessels is required. The culture of plant cells in bioreactors also has various constraints like genetic instability of cell lines, shear sensitivity, low yields, slow growth and, most importantly, scale-up problems [5]. Genetically transformed hairy roots generated because of infection of *Agrobacterium rhizogenes* in plant cells provide an alternative route for secondary metabolite production.

The benefits that come along with the use of genetically transformed hairy root have made it a promising system for secondary metabolite production. Hairy roots are often known to grow much faster than plant cell cultures and do not require phytohormones in the medium for growth [6]. They often possess a greater biosynthetic capacity for the production of secondary metabolite in comparison to their mother plants [7]. The hairy roots lack geotropism, show lateral branching and are genetically more stable [8]. It can also serve the purpose of being a model system for the study of plant metabolism and physiology [9].

For several decades, hairy root cultures have been explored for their potential to produce various valuable metabolites that are present in wild type roots. Moreover, genetic engineering of secondary metabolism by inserting useful genes can help to increase the biosynthetic capacity and could produce multiple secondary compounds [10].

2. CHALLENGES IN DESIGNING BIOREACTORS FOR HAIRY ROOTS

The main motivation behind using hairy root cultures for production of bioactive molecules is their genetic stability, large biomass and higher product concentration [11], but the lack of technology for commercial exploitation of these roots is the major bottleneck in their use. Thus, there is a necessity to identify an appropriate scalable bioreactor configuration to expedite mass production of hairy roots culture based secondary metabolites. Bioreactors are self-contained systems with a sterile environment designed to optimise and monitor as well as provide homogenous culture environment conditions such as pH, dissolved gases, aeration, and temperature along with in/outflow channels of liquid and air for mass propagation of cells, tissues, somatic embryos or organogenic propagules [10].

A variety of bioreactor configurations have been used by various research groups, but it has been observed experimentally that the scaling up of cultivation of hairy root from shake flask to industrial scale is an arduous task due to the intricate morphology and non-uniform growth possessed by the hairy roots [12].

The complex fibrous nature of hairy root growth poses distinctive limitations while scaling up in bioreactors. The hairy roots form an interlocked network which resists mass transfer to nutrient and oxygen forming non-homogenous culture environment thus giving rise to a pack of senescent tissues [13]. Oxygen deficiency is one of the principal challenges in mass production of hairy root as it is the growth-limiting factor in the hairy root bioreactors. Development of oxygen gradients is a common phenomenon observed in hairy root bioreactors and a slight decrease in the dissolved oxygen concentration negatively affects growth rate and thus the synthesis of certain secondary metabolites [7].

Moreover, the hairy root morphology is very sensitive as the roots respond to the little changes in the local environmental conditions like temperature and shear stress. Any change in the morphology of hairy root such as density, thickness and root length, significantly affect the production of secondary metabolite [14]. Hairy root cultures are very delicate and fragile in nature. Although vigorous mixing by rotating impellers can augment mass transfer, the consequent increase in hydrodynamic shear stress reduces the vitality of the hairy root tissue and leads to the formation of callus. Since these cultures cannot withstand the direct encounter with the rotating impeller in the bioreactor, isolating the growing roots from the moving impeller in the bioreactor could be helpful [15]. Some species of hairy root demand for

Table 1. Desirable properties in a bioreactor for hairy root culture.

The Properties of an Ideal Bioreactor for Hairy Root Production
• It should impose minimum hydrodynamic shear stress.
• It should maintain a contamination free environment.
• If the cultures are mixotropic or phototropic, there should be efficient arrangement of light and CO_2 for photosynthesis.
• It should provide a homogenous culture environment for steady and continuous production of bioactive molecules.
• It should not have a restriction to the nutrient availability as well as nutrient or oxygen delivery to the biomass (no mass transfer limitation).
• It should consider the requirement of support matrix for roots.

perfect aseptic conditions for longer periods for relevant growth [12].

While scaling up, as the volume increases, the pressure on hairy roots due to their own weight also increases. Thus container size and volume of medium in the bioreactor for growth are also important factors to be considered [16]. On-line monitoring of biomass growth, product formation substrate utilization and other culture conditions is a major challenge for hairy root growth in bioreactors. Indirect methods like using change in conductivity of medium for estimating biomass increase can be used [17]. The recent design of bioreactors for hairy root cultures now considers factors such as the requirement of a support matrix for biomass production [14]. Light is also an important factor that affects cells in the phase in which they produce pigments. Light exposure to the culture affects the activity of enzymes which produce pigments such as in case of light-dependent secondary metabolites [18]. However, all hairy roots do not require light to produce the bioactive compounds.

Hence, an ideal bioreactor for hairy root culture should have contradicting properties like high gas and liquid mass transfer coefficient and low shear stress to roots (Table **1**). Developing such a reactor having all the desirable properties for large scale culture of hairy roots is a major challenge.

3. BIOREACTORS FOR HAIRY ROOT CULTIVATION FOR THE SECONDARY METABOLITE PRODUCTION

Different bioreactor configurations have been used for large scale culture of hairy roots (Table **2**). Kim *et al.*, (2002) classified the bioreactors for hairy roots into three main types: the liquid phase reactors, the gas phase reactors, or the hybrid reactors (a combination of both). Similarly, bioreactor for hairy root cultures can also be classified as agitated reactors and bed reactors (Fig. **2**) [19]. Liquid phase reactors are those reactors in which the hairy root biomass in completely submerged in the liquid media. In these reactors, mass transfer of gaseous media is rate limiting. Stirred tank reactors, bubble column reactors, air lift reactor, submerged convective flow reactors *etc*. are some examples of liquid phase reactors. Gas phase reactors include trickle bed reactor, droplet phase reactor, nutrient mist reactor *etc.* In these reactors, hairy root biomass is not submerged in liquid and the roots are exposed to air and mixture of air and liquid media. The rate of supply of liquid media in these reactors is important and should be precisely controlled. In gas phase reactors, liquid phase mass transfer of oxygen and other nutrients in significantly enhanced. However, uniform distribution of root tips in the reactor is a major problem in these reactors. Therefore, it is suggested to initially grow the root tips in liquid phase system until the root tips are distributed uniformly throughout the packing matrix and then run the reactor in the gas phase [20]. In the upcoming text in this review, we have discussed the merits and demerits of some reactors used for cultivation of hairy roots.

3.1. Stirred Tank Reactors

Stirred tank reactors are conventional reactors known for efficient mass and heat transfer properties. These reactors have an impeller run by a motor which provides energy for mixing. Although the impeller gives high mass transfer rates, it causes high shear stress on the roots. Hairy roots are highly sensitive to shear stress as they change their morphology at high shear stress. High shear also causes injury to roots causing callus formation. Therefore, several modifications have been proposed in stirred tank reactors and mostly modified stirred tank reactors are used for hairy root cultures (Fig. **3**).

Hairy roots cultivation of *Azadirachta indica* was compared with different liquid phase bioreactor configurations (stirred-tank reactor, bubble column reactor and modified bubble column reactor) for mass production of the metabolite Azadirachtin. After 25 days, no hairy root and high phenolic content indicated that there was a high amount of shear stress on the roots from impellers of conventional stirred tank reactors [21]. The impeller should be operated with reduced power input and speed for the culturing of hairy roots in order to prevent them from damage because of their highly shear sensitive nature. High-speed impeller damages the roots and causes callus formation. Small modifications in the conventional design can significantly reduce the shear stress on the roots and enhance the biomass growth. A 14L STR was modified using a stainless steel cage to segregate the roots from the stirrer. A production rate of 8.2 mg hyoscyamine per liter per day was achieved by growing the roots in modified stirred tank reactor at 30°C in continuous mode [22]. A mass production strategy of hairy root of *Artemisia annua* for an important drug Artemisinin using a 3L modified STR had been established. The reactor utilised steric impeller for lower shear stress, a sintered sparger for aeration and a perforated Teflon mesh for segregation, which allowed

Table 2. Summary of some bioreactors used for hairy root cultures.

Sr. No.	Metabolite	Source	Bioreactor(s)	Volume (ml)	Refs.
1.	Anisodamine	*Hyoscyamus niger*	Bubble-column bioreactor	1500	[27]
	Scopolamine				
	Hyoscyamine		Hybrid bubble-column/spray bioreactor	1500	
	Cuscohygrine				
2.	Saponin	*Talinum paniculatum*	Balloon-type bubble bioreactor (BTBB)	1000	[55]
3.	Artemisinin	*Artemisia annua*	Modified stirred tank	3000	[15]
4.	Artemisinin	*Artemisia annua*	Modified bubble column reactor	3000	[12]
			Nutrient mist bioreactor	5000	
			Modified nutrient mist bioreactor	5000	
5.	Artemisinin	*Artemisia annua*	Modified stirred tank	3000	[23]
6.	Ajmalicine	*Catharanthus roseus*	Bubble column	3000	[31]
			Rotating drum bioreactor	7000	
			Modified Bubble column with polypropylene (PP) mesh support	5000	
			Modified Bubble column with Polyurethane foam (PUF) support	5000	
7.	Scopolamine	*Brugmansia candida*	Modified stirred tank	1500	[56]
	Anisodamine				
	Hyoscyamine				
8.	Azadirachtin	*Azadirachta indica*	Stirred-tank	3000	[21]
			Bubble column	3000	
			Bubble column with polypropylene basket	3000	
			Bubble column with polyurethane foam disc	3000	
9.	Betalaine	*Beta vulgaris*	Bubble column reactor with elicitor	3000	[32]
10.	Betacyanin	*Beta vulgaris*	Airlift bioreactors (cone, balloon, bulb, drum and column bioreactors)	5000	[18]
11.	Puerarin	*Pueraria phaseoloides*	airlift bioreactors	2500	[33]
12.	Shikonin	*Lithospermum erythrorhizon*	Two Phase Bubble column reactor	1500	[30]
13.	-	*Fragaria ananassa* Duch	Mist Bioreactor	4000	[42]
			Droplet Bioreactor	4000	
			Air-sparged bioreactor	1500	
14.	Diterpenoids	*Salvia sclarea*	Nutrient sprinkle bioreactor	10000	[44]
15.	Artemisinin	*Artemisia annua* L	Modified inner-loop airlift bioreactor	2500	[34]
16.	Cichoric acid	*Echinacea purpurea*	Modified airlift bioreactor	1700	[35]
17.	Hypericin	*Hypericum perforatum* L.	Balloon type bubble bioreactor (BTBB)	3000	[57]
18.	Ginsenoside	Panax quinquefolium	Nutrient sprinkle bioreactor	10000	[58]

(Table 2) Contd….

Sr. No.	Metabolite	Source	Bioreactor(s)	Volume (ml)	Refs.
19.	Glycyrrhizin	*Glycyrrhiza glabra*	Air sparged and mechanically agitated bioreactor	5000	[59]
20.	Artemisinin	*Artemisia annua* L	Nutrient mist bioreactor.	2300	[43]
			Inner-loop nutrient mist bioreactor	2300	
			Modified innerloop nutrient mist bioreactor	2300	
21.	Hyoscyamine	*Datura stramonium*	Modified stirred tank reactor	140000	[22]
22.	Azadirachtin	*Azadirachta indica*	Modified stirred tank with PUF and steric impeller	3000	[24]
23.	Betaxanthin	*Beta vulgaris* L.	Bubble column reactor	2000	[25]
	Betacyanin				
24.	Esculin	*Cichorium intybus* L	Bubble column reactor	1750	[26]
	Esculetin		Nutrient sprinkle reactor	1000	
			Acoustic mist reactor	300	
25.	-	*Artemisia annua*	Bubble column reactor	1500	[28]
			Mist reactor	1500	
26.	-	*Artemisia annua*	Bubble column reactor	1500	[8]
			Nutrient mist reactor	1500	
27.	-	*Atropa belladonna*	Segmented bubble column reactor	2500	[29]
28.	Betacyanins	*Beta vulgaris*	Bubble column reactor	3000	[16]
	Betaxanthins				
29.	Scopolamine	*Duboisia leichhardtii hairy*	Airlift reactor	3000	[36]
30.	Camptothecin	*Ophiorrhiza pumila*	Modified airlift reactor	3000	[37]
31.	L-DOPA	*Stizolobium hassjoo*	Mist trickling reactor	3000	[41]
32.	-	*Beta vulgaris Carthamus tinctorious*	Mist rotating drum	1400	[49]
33.	Azadirachtin	*Azadirachta indica*	Stirred tank reactor	3000	[14]
			Bubble column reactor	3000	
			Nutrient spray reactor	4000	
			Nutrient mist reactor	4000	

operation at higher RPM and better mass transfer. After 28 days, 18.52 g/L of biomass 4.63 mg/L of artemisinin concentration and 10.33 mg/L of artemisinin using an elicitor (methyl jasmonate) was obtained in the bioreactor [15]. A successful scale-up of artemisinin production by hairy roots of *Artemisia annua* from shake flasks to a 3L stirred tank reactor with setric impeller was obtained under optimised conditions thereby accumulating 6.3 g/L dry weight (37.50 g fresh weight) of biomass and artemisinin content of 0.32 mg/g after 25 days of batch cultivation [23]. In another experiment, a study was conducted for large scale azadirachtin production by hairy root cultivation of *Azadirachta indica* in a modified stirred tank reactor under optimized culture conditions. In order to facilitate highly dense cultivation of *A. indica* hairy roots, a 3L reactor with steric impeller and polyurethane foam (PUF) disc as support for self-immobilisation of hairy roots had been used. 6.4 mg/g (97.28 mg/L) of azadirachtin and 15.2 g/L of biomass were produced after 25 days of batch cultivation mode [24].

3.2. Bubble Column Reactor

This type of bioreactor is a pneumatically agitated liquid phase bioreactor consisting of a vertical column where the hairy roots are immersed in the medium and the mixing/agitation is carried out by the up-flow movement of air bubbles from a sparger located at the bottom of the reactor (Fig. **4**). These are more suitable as they impose less shear stress on the roots as compared to mechanically agitated reactors. Pigment production and biomass accumulation of hairy root of Beta vulgaris were tested for scale-up in a 2 L bubble column reactor and mass transfer limitation and

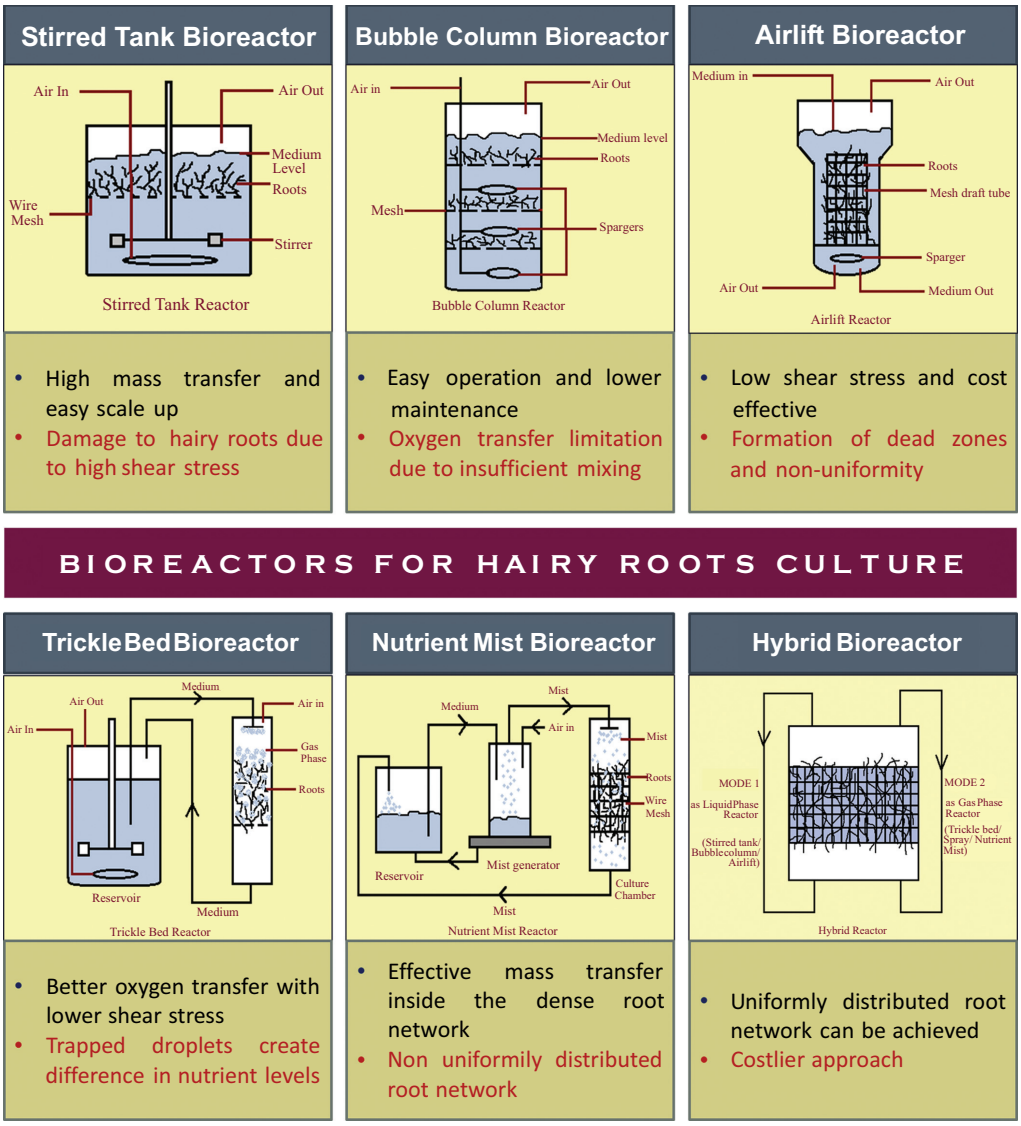

Fig. (2). Classification of bioreactors commonly used for hairy root cultures.

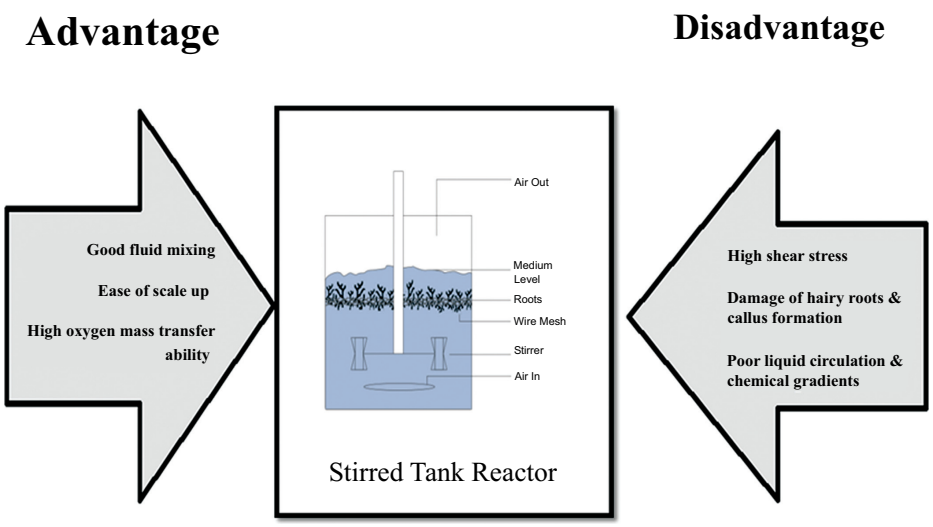

Fig. (3). Advantages and disadvantages associated with Stirred Tank Bioreactors.

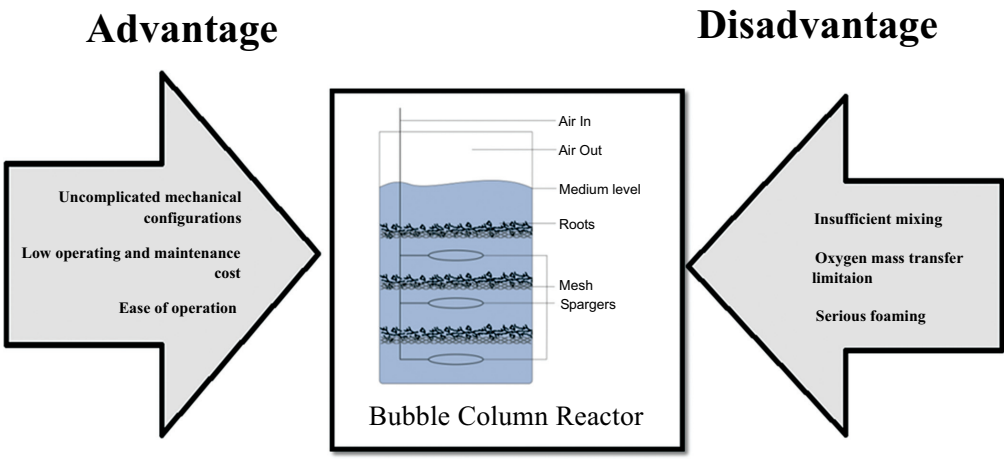

Fig. (4). Advantages and disadvantages associated with Bubble Column Bioreactors.

lower yields than shake flasks were observed [25]. Hairy root culture of *Cichorium intybus* L. had been examined for scale-up in a bubble column bioreactor with a vertical plastic basket for root immobilization. This was an efficient method to maintain a nearly even distribution of roots over the height of the vessel, and enabled a multidirectional root growth pattern [26]. *Hyoscyamus niger* hairy roots were evaluated for scale-up in bubble column bioreactors, owing to the simplicity and reliability of the reactor. The vessel was integrated with a stainless steel mesh as inoculation support. The concentrations of secondary metabolites scopolamine, hyoscyamine and cuscohygrine produced in bubble column reactor by *H. niger* hairy roots were found to be 5.3, 1.6 and 26.5 mg/g dry wt, respectively [27]. Jaremicz *et al.* studied the growth dynamics of the hairy root culture of *Artemisia annua* in bubble column reactor and other culture systems [28]. The specific growth rate and the specific sugar consumption rate were calculated and biomass yields from sugar and maintenance coefficients for sugar were obtained using three different culture systems. In another study, hairy roots of *Artemisia annua* grown in the bubble column reactor observed 15.3 g DW/L biomass concentration which was better than the mist reactor [8]. A 2.5 L segmented bubble column reactor was tested for the hairy roots cultures of *Atropa belladonna* for the assessment of growth kinetics, stoichiometry, and production of atropine metabolite. The reactor was divided into vertical segments by incorporating the mesh screens and each segment consisted of a sparger [29]. The production of betacyanins and betaxanthins was monitored in a modified bubble column reactor by the hairy root culture of red beets with two-step basket where air was continuously bubbled. It produced about 1.2 times higher biomass compared to one step basket [16]. Sim, Chang, & Town, (1993) proposed a two-phase bubble column reactor for hairy roots of *Lithospermum erythrorhizon* for shikonin production and obtained 572.6 mg/L shikonin and 15.6 g/L biomass after a period of 54 days. Cultivation of *Azadirachta indica* hairy roots for large-scale production of secondary metaboloite azadirachtin was carried out in a conventional bubble column reactor as well as modified bubble column reactors. Bubble column was modified with the incorporation of polypropylene basket in one case, and with polyurethane foam disc as root supports in another. The result showed that modified reactor configurations facilitated higher biomass and metabolite production as compared to a conventional bioreactor, and bubble column with polyurethane foam being the most suitable configuration [21]. Recently, in one more study, the bubble column reactor was modified with polypropylene (PP) mesh support & with Polyurethane foam (PUF) support to produce Ajmalicine by transformed roots of *Catharanthus roseus*. Modified reactor with PUF support accumulated 34 ± 2.3 mg/L of ajmalicine which accounted for a productivity of 1.13 mg/L [31]. Suresh, Thimmaraju, Bhagyalakshmi, & Ravishankar (2004) investigated the influence of elicitors, polyamine and methyl jasmonate, for the production of betalain in hairy root cultures of Beta vulgaris grown utilising a 3 L bubble column reactor with a plastic basket for anchorage.

3.3. Airlift Reactors

These are similar to bubble column reactors as the method mass and energy input in airlift reactors are also achieved pneumatically without any mechanical agitation but in contrast, possess significantly lower shear stresses than in stirred tank reactors. Advantages and disadvantages of airlift reactors are discussed in Fig. (**5**). The difference between bubble column and airlift reactor is that an airlift reactor makes use of a draft tube. This helps to separate the upward and downward flow as the riser and down-comer components. The density difference between riser and down-comer facilitates the liquid to circulate with high turbulence. Successful scale-up of puerarin production has been achieved from the hairy roots of *Pueraria phaseoloides* in a 2.5 L airlift bioreactor. After a run of about three weeks, 5570 µg/g dry wt puerarin was accumulated which was 200 times as a 250ml shake flask culture [33]. A transformation of conventional type was done as modified inner loop airlift reactor (MILAB), fitted with stainless steel meshes along the height of the column, and was utilised to enhance the production of artemisinin by *Artemisia annua* hairy roots by optimising the culture environment conditions and yielded 577.5 mg/L of artemisinin after 20 days [34]. Another transformation of the conventional type was done by the introduction of stainless steel mesh (average pore size 700 µm) as the draft tube to produce cichoric acid from hairy root cultures of Echinacea. Homogenous Biomass distribution with cichoric acid production of 178.2 ± 4.9 mg/L at day 30 could be achieved at

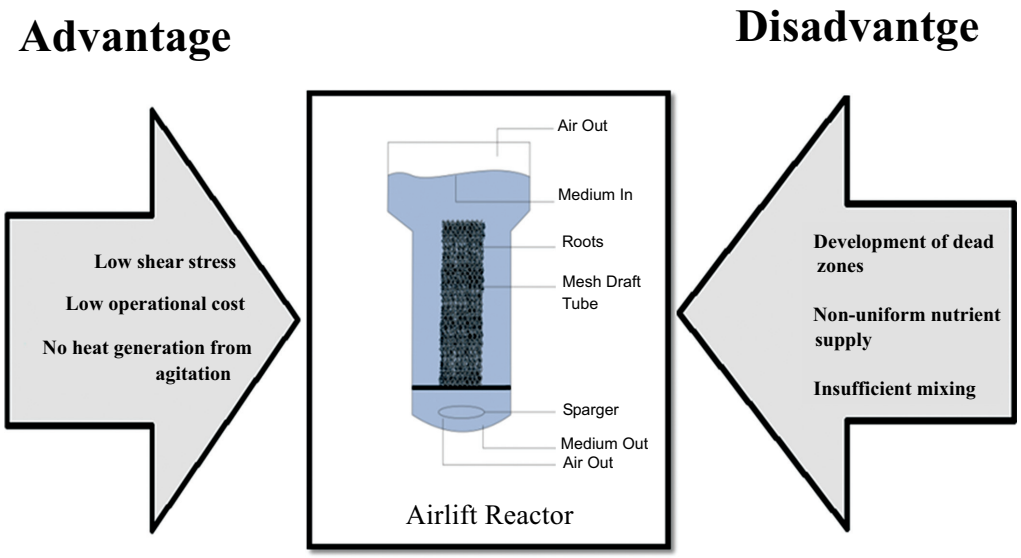

Fig. (5). Advantages and disadvantages associated with Air Lift Bioreactors.

an aeration rate of 0.012m3 h^{-1} and 6 min of ultrasound treatment [35]. Similarly, scopolamine density increased from 0.5g/L to 1.3g/L during 11 weeks of culture of hairy roots of *Duboisia leichhardtii* [36] & 8.7 ± 1.3 mg/L of camptothecin by HRs of *Ophiorrhiza pumila* in 8 weeks [37] in the airlift reactor when polyurethane foam or stainless steel mesh was used as aid.

The configuration of the airlift bioreactor used also concluded the production of metabolite from the hairy culture. Amongst the configurations of cone, balloon, bulb, drum and column type of airlift bioreactors (5 L) examined for hairy roots of *Beta vulgaris* L. (red beet), the cone type gave the best betacyanin accumulation [18].

Computational fluid dynamics (CFD) simulation is a method which helps us to explore the hydrodynamic behaviour and to obtain optimised reactor designs. Recently, Liu *et al.* used CFD model to optimise the height to diameter ratio and the position to install the ultrasound transducers in an airlift reactor to augment oxygen mass transfer for the culture of hairy roots [38, 39].

3.4. Liquid-dispersed/Bed Reactors

Liquid dispersed or gas phase or bed reactors are the category of reactors in which a mixture of ambient air and the nutrient medium is dispersed or trickled on the bed of roots in spray mode or mist mode or droplets mode and the unused medium is collected and re-circulated. The major advantage of these reactors is that they overcome the problem of oxygen mass transfer to a greater extent and also facilitate a lower shearing stress environment suitable for cultivation of hairy roots in comparison to the liquid phase reactors. Moreover, it is often observed in the liquid phase reactors that a fraction of liquid medium gets entrapped inside the root network which is depleted of oxygen and nutrient [40, 41]. However, there is a requisite of support for the hairy root culture in the form of matrix or mesh.

Nutrient mist, droplet, nutrient sprinkle, trickle-bed or spray reactors [27, 41-44], are the various types of liquid dispersed reactors differentiated on the basis of the size of droplets. The droplet sizes for the mist reactors are usually micron scale 0.01-10 μm and more than 10 μm for other types [45].

Artemisia annua hairy roots showed a different transient growth when compared in bubble column reactor (liquid phase) and trickle bed reactor (gas phase) [8, 46]. The conventional design of trickling bed reactor was modified to create a novel reactor by using mesh as a hindrance known as mesh hindrance mist trickling bioreactor (MHMTB) (Fig. **6**). This mesh acts as a barrier and allows lateral root bridging for the production of L-DOPA from *Stizolobium hassjoo* hairy roots [47]. In another study, production of diterpenoids from the hairy root cultures of *Salvia sclarea* was done using a 10 L nutrient sprinkle bioreactor during a time period of 30 days [44].

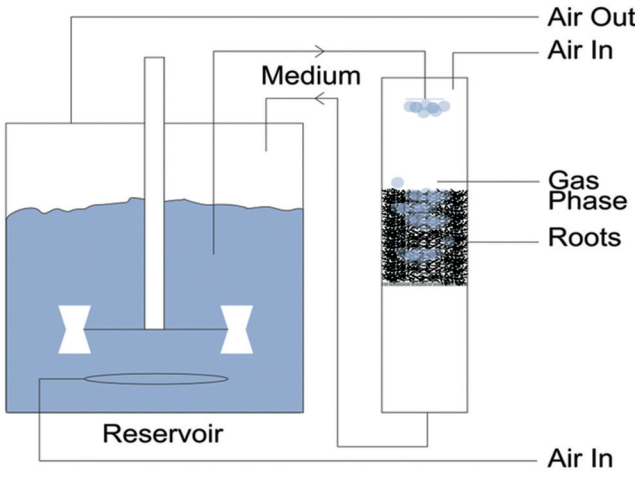

Fig. (6). Trickle bed bioreactor.

A mist reactor generally consists of the following components along with the reactor: the heating system, the oxygen

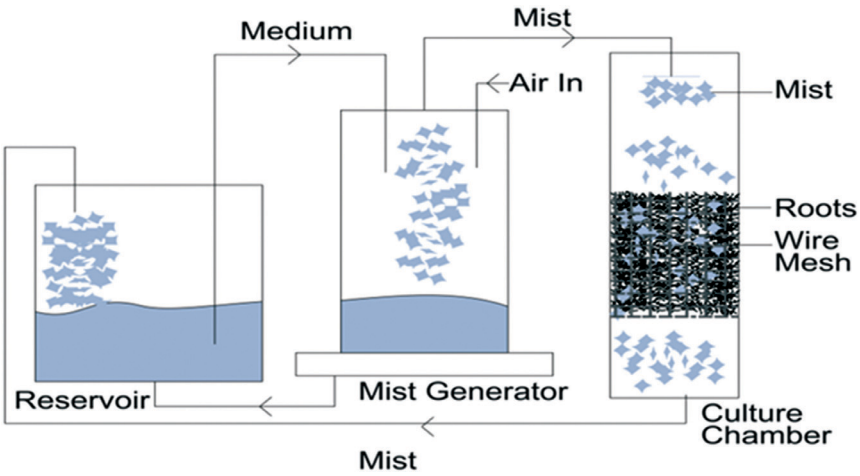

Fig. (7). Nutrient Mist Bioreactor.

flow control system and the mist generator system (Fig. **7**). The heating system consists of a polymer thick film (PTF) heater for the heating as it has a rapid temperature switching effect. The oxygen flow control system element works for regulating the oxygen flow and the mist generator system creates a spray of mist. Finally, these components are connected to the mist inducer and reactor [43, 48].

In a nutrient mist reactor, the medium is passed in the form of a fine mist which has a large surface area, therefore, higher oxygen mass transfer. This reactor is advantageous over other reactors for hairy root cultivation. This reactor is able to accommodate an environment for fast replenishment of the nutrients as well as help in removing toxic by-products. There is also low shear stress due to the absence of an agitator. Nutrient mist reactor had been used for the culturing of roots of *Beta vulgaris* and *Carthamus tinctorius* with a nylon support to evaluate the various parameter of misting cycle, size of inoculum, batch or continuous operation and sugar content [49]. *Agrobacterium rhizogenes* mediated transformed roots of *Azadirachta indica* for azadirachtin (a biopesticide) production was accomplished in gas-phase reactors (nutrient spray and mist bioreactor) to deduce the possibility of scale-up of *Azadirachta indica* hairy root culture. 9.8 g/L dry weight of biomass and azadirachtin accumulation of 2.8 mg/g biomass (27.4 mg/L) in 25 days of batch cultivation period was obtained [14]. The possibility of scale-up of a nutrient mist reactor from 1 L to 20 L without affecting the biomass and secondary metabolite production was examined using hairy root cultures of *Artemisia annua* L. and *Arachis hypogaea* (peanut). The results showed that *A. hypogaea* growth in 1 L reactors was $\mu = 0.173$/day with biomass yield of 12.75 g (DW)/L, which exceeded that in shake flasks at $\mu = 0.166$/day and 11.10 g (DW)/L. The most possible growth rate and biomass yield in 20 L reactor were $\mu = 0.147$ and 7.77 g (DW)/L only when the medium flow rate delivery was enhanced [50].

Mathematical modelling of a recent approach offers an effective method to study the reaction parameters. Osama, (2013) in order to deduce the culture parameters and operating conditions *viz*, size of inoculum, mist ON/OFF time, initial packing density, media volume, initial sucrose concentration in media and time of culture, used the Artificial Neural Network (ANN) model approach. ANNs are based on the biological neural network. Similarly, a mathematical model has been developed to improvise the ON/OFF mist duty cycle for the hairy root culture. The ON/OFF cycle provides a way to control the accessibility and transport rate of nutrients to the hairy roots in the reactor [51, 52].

Various analogous studies have been performed to get the most favourable bioreactor configuration for hairy roots cultivation. In a comparative study for the growth of hairy root of *Artemisia annua* between the mist reactor and the bubble column reactor, the biomass yield was three times in mist reactor as in bubble column [46]. Similarly, when another study between an air-sparged bioreactor (control), a droplet bioreactor and a mist bioreactor was done for hairy roots of strawberry (*Fragaria ananassa* Duch.), the biomass yields were higher in the mist reactor [42]. Similarly, another comparison between stirred tank, bubble column, nutrient spray, and nutrient mist reactor for *Azadirachta indica* hairy roots for azadirachtin production showed that the nutrient mist reactor was the best option [14].

3.5. Hybrid Reactors

Although the gas phase reactors offer more benefits over the liquid phase reactors, there are also some limitations associated with the gas phase reactors. In order to distribute the roots uniformly in gas phase reactors, manual loading is required. The concept of a hybrid reactor comes with a solution to this limitation of gas phase reactor. The strategy is the combination of liquid-phase and gas-phase reactor system for the most effective hairy root cultivation. A combination of bubble column and trickle bed reactor was proposed as the best compromise by [53]. Ramakrishnan and Curtis, [54] also used a hybrid reactor that operated in bubble column mode initially for suspension, distribution and attachment of roots to the packing rings in the reactor. Following this, roots were operated in the gas phase reactor *i.e.*, trickle bed mode, after two weeks.

A bubble column and nutrient mist hybrid bioreactor system of working volume 1.5 L was used to examine the transient growth of *Artemisia annua* hairy roots. The reactor was first run in bubble column mode, and later after 6 days,

all the media in the reactor was drained and it was run in nutrient mist mode. An air flow rate was kept at 5 L/min that helped in transporting the mist and flow rate of the nutrient medium was 1.1 ± 0.1 mL/min. The mist cycle was set for 5 min on followed by 15 min off. The highest observed final biomass concentrations were 15.3 g DW/L for the bubble column reactor and 14.4 g DW/L for the mist reactor [8, 28].

Recently, the production of tropane alkaloids from the hairy roots of *Hyoscyamus niger* was studied in bubble-column/spray bioreactors hybrid system. The hybrid reactor has a working volume of 1500ml and was initially run in bubble column mode for 7 days, followed by spraying mode. The medium flow rate was 100 ml/min dispersed using the 1.5 size TN-type hydraulic nozzle (Spraying Systems Co, Wheaton, US-IL), at regular intervals of 10 min intervals. Aeration was done at constant 0.8 vvm. The hybrid reactor used was made of glass and was incorporated with one drain valve, one medium reservoir and also a multi-tier stainless steel scaffold consisting of rods and 1 mm mesh straps (3, 9, 6 mm). The alkaloid anisodamine gave a concentration of 0.67 mg/g dry wt in the hybrid reactor which was 3.5 fold greater than the bubble column reactor. The alkaloids scopolamine, hyoscyamine and cuscohygrine were also evaluated [27].

4. CONCLUSION

Hairy roots offer a very promising approach for the successful production of valuable bioactive compounds. The stability, hormone free medium requirements and fast growth of hairy roots make it suitable to be utilised for the production and extraction of high value metabolites. However, the large scale cultivation has many challenges due to the morphology and delicate nature of hairy roots. Although many efforts have been done to obtain a bioreactor configuration for highest biomass possible, there is a still a need to get the most suitable bioreactor which provides high oxygen mass transfer, better growth characteristics, homogenous culture environment and minimum shear stress. Nutrient mist reactors have proven to be a suitable bioreactor configuration but there is need to design higher volume NMRs alongside considering the economy. The aspect of future use of hairy roots for commercialisation is mainly dependent on the low capital as well as operating cost. Recent developments of past years of mathematical modelling and computational fluid dynamics are magnificent.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors are thankful to Integral University, Lucknow for providing the necessary support and infrastructure to conduct this study. We declare that there is no conflict of interest in this work.

REFERENCES

[1] Tepfer D. Genetic transformation using *Agrohacterium rhizogenes*. Physiol Plant 1990; 79(1): 140-6.

[2] Bensaddek L, Villarreal ML, Fliniaux M. Induction and growth of hairy roots for the production of medicinal compounds. Electron J Integr Biosci 2008; 3(1): 2-9.

[3] Biswas B, Scott PT, Gresshoff PM. Tree legumes as feedstock for sustainable biofuel production: Opportunities and challenges. J Plant Physiol 2011; 168(16): 1877-84.

[4] Ramachandra RS, Ravishankar GA. Plant cell cultures: Chemical factories of secondary metabolites. Biotechnol Adv 2002; 20(2): 101-53.

[5] Bourgaud F, Gravot A, Milesi S, Gontier E. Production of plant secondary metabolites: A historical perspective. Plant Sci 2001; 161: 839-51.

[6] Rao RS, Ravishankar G. Plant cell cultures: Chemical factories of secondary metabolites. Biotechnol Adv 2002; 20(2): 101-53.

[7] Srivastava S, Srivastava AK. Hairy root culture for mass-production of high-value secondary metabolites. Crit Rev Biotechnol 2007; 27(1): 29-43.

[8] Kim YJ, Weathers PJ, Wyslouzil BE. Growth of Artemisia annua hairy roots in liquid- and gas-phase reactors. Biotechnol Bioeng 2002; 80(4): 454-64.

[9] Hu Z, Du M. Hairy root and its application in plant genetic engineering. J Integr Plant Biol 2006; 48(2): 121-7.

[10] Stiles AR, Liu C. Hairy root culture : Bioreactor design and process intensification. In: *Biotechnology of Hairy Root Systems*. Springer, Berlin, Heidelberg; 2013: 91-114.

[11] Paek KY, Murthy HN, Zhong JJ. Production of biomass and bioactive compounds using bioreactor technology. Springer: Berlin, 2014.

[12] Patra N, Srivastava AK. Artemisinin production by plant hairy root cultures in gas- and liquid-phase bioreactors. Plant Cell Rep 2016; 35(1): 143-53.

[13] Vashishtha M, Sharma N. Nutrient mist reactor : A remarkable new approach. Int J Adv Sci Eng Technol 2015; 3(1): 8-11.

[14] Srivastava S, Srivastava AK. *In vitro* Azadirachtin production by hairy root cultivation of *Azadirachta indica* in nutrient mist bioreactor. Appl Biochem Biotechnol 2012; 166(2): 365-78.

[15] Patra N, Srivastava AK. Enhanced production of artemisinin by hairy root cultivation of *Artemisia annua* in a modified stirred tank reactor. Appl Biochem Biotechnol 2014; 174(6): 2209-22.

[16] Neelwarne B, Thimmaraju R. Bioreactor for cultivation of red beet hairy roots and *in situ* recovery of primary and secondary metabolites. Eng Life Sci 2009; 9(3): 227-38.

[17] Goswami M, Akhtar S, Osama K. Strategies for monitoring and modeling the growth of hairy root cultures: An *In Silico* Perspective. In: *Hairy Roots*. Singapore: Springer Singapore; 2018: 311-327.

[18] Shin KS, Murthy HN, Ko JY, Paek KY. Growth and betacyanin production by hairy roots of *Beta vulgaris* in airlift bioreactors. Biotechnol Lett 2002; 24(24): 2067-9.

[19] Eibl R, Eibl D. Bioreactors for plant cell and tissue cultures. In: *Plant Biotechnology and Transgenic Plants*. Marcel Dekker, Inc.; 2002: 163-199.

[20] Ramakrishnan D, Salim J, Curtis WR. Inoculation and tissue distribution in pilot-scale plant root culture bioreactors. Biotechnol Tech 1994; 8: 639-44.

[21] Srivastava S, Srivastava AK. Production of the biopesticide azadirachtin by hairy root cultivation of *Azadirachta indica* in liquid-phase bioreactors. Appl Biochem Biotechnol 2013; 171(6): 1351-61.

[22] Hilton MG, Rhodes MJC. Growth and hyoscyamine production of "hairy root" cultures of Datura stramonium in a modified stirred tank reactor. Appl Microbiol Biotechnol 1990; 33(2): 132-8.

[23] Patra N, Kumar SA. Mass scale artemisinin production in a stirred tank bioreactor using hairy roots of *Artemisia Annua*. Int J Biosci Biochem Bioinforma 2014; 4: 467-74.

[24] Srivastava S, Srivastava AK. Azadirachtin production by hairy root cultivation of *Azadirachta indica* in a modified stirred tank reactor. Bioprocess Biosyst Eng 2012; 35(9): 1549-53.

[25] Mukundan U, Carvalho EB, Curtis WR. Growth and pigment production by hairy root cultures of *Beta vulgaris* L. in a bubble column reactor. Biotechnol Lett 1998; 20(5): 469-74.

[26] Bais HP, Suresh B, Raghavarao KSMS, Ravishankar GA. Performance of hairy root cultures of *Cichorium intybus* L. in bioreactors of different configurations. Vitr Cell Dev Biol Plant 2002; 38(6): 573-80.

[27] Jaremicz Z, Luczkiewicz M, Kokotkiewicz A, Krolicka A, Sowinski P. Production of tropane alkaloids in *Hyoscyamus niger* (black henbane) hairy roots grown in bubble-column and spray bioreactors. Biotechnol Lett 2014; 36(4): 843-53.

[28] Kim YJ, Weathers PJ, Wyslouzil BE. Growth dynamics of Artemisia annua hairy roots in three culture systems. Biotechnol Bioeng 2003; 83(4): 428-43.

[29] Kwok KH, Doran PM. Kinetic and stoichiometric analysis of hairy roots in a segmented bubble column reactor. Biotechnol Prog 1995; 11(4): 429-35.

[30] Sim SJ, Chang HN, Town DS. Biotechnology 1993; 1(2): 145-50.

[31] Thakore D, Srivastava AK, Sinha AK. Mass production of Ajmalicine by bioreactor cultivation of hairy roots of *Catharanthus roseus*. Biochem Eng J 2017; 119: 84-91.

[32] Suresh B, Thimmaraju R, Bhagyalakshmi N, Ravishankar GA. 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 2004; 39(12): 2091-6.

[33] Kintzios S, Makri O, Pistola E, Matakiadis T, Ping Shi H, Economou A. Scale-up production of puerarin from hairy roots of Pueraria phaseoloides in an airlift bioreactor. Biotechnol Lett 2004; 26(13): 1057-9.

[34] Liu C, Wang Y, Guo C, Ouyang F, Ye H, Li G. Enhanced production of artemisinin by *Artemisia annua* L hairy root cultures in a modified inner-loop airlift bioreactor. Bioprocess Eng 1998; 19(5): 389-92.

[35] Abbasi BH, Liu R, Saxena PK, Liu CZ. Cichoric acid production from hairy root cultures of *Echinacea purpurea* grown in a modified airlift bioreactor. J Chem Technol Biotechnol 2009; 84(11): 1697-701.

[36] Muranaka T, Ohkawa H, Yamada Y. Continuous production of scopolamine by a culture of *Duboisia leichhardtii* hairy root clone in a bioreactor system. Appl Microbiol Biotechnol 1993; 40(2-3): 219-23.

[37] Sudo H, Yamakawa T, Yamazaki M, Aimi N, Saito K. Bioreactor production of camptothecin by hairy root cultures of *Ophiorrhiza pumila*. Biotechnol Lett 2002; 24(5): 359-63.

[38] Liu R, Liu Y, Liu CZ. Development of an efficient CFD-simulation method to optimize the structure parameters of an airlift sonobioreactor. Chem Eng Res Des 2013; 91(2): 211-20.

[39] Williams GRC, Doran PM. Hairy root culture in a liquid-dispersed bioreactor: Characterization of spatial heterogeneity. Biotechnol Prog 2000; 16(3): 391-401.

[40] Kim Y, Wyslouzil BE, Weathers PJ. Secondary metabolism of hairy root cultures in bioreactors. Vitr Cell Dev Biol - Plant 2002; 38(1): 1-10.

[41] Huang SY, Hung CH, Chou SN. Innovative strategies for operation of mist trickling reactors for enhanced hairy root proliferation and secondary metabolite productivity. Enzyme Microb Technol 2004; 35(1): 22-32.

[42] Nuutila M, Kauppinen V. Growth of hairy root cultures of strawberry (Fragaria¥ ananassa Duch.) in three different types of bioreactorsBiotechnol Tech 1997; 11(6): 363-6.

[43] Liu CZ, Wang YC, Zhao B, *et al.* Development of a nutrient mist bioreactor for growth of hairy roots. Vitr Cell Dev Biol - Plant 1999; 35(3): 271-4.

[44] Kuźma Ł, Bruchajzer E, Wysokińska H. Methyl jasmonate effect on diterpenoid accumulation in *Salvia sclarea* hairy root culture in shake flasks and sprinkle bioreactor. Enzyme Microb Technol 2009; 44(6-7): 406-10.

[45] Weathers P, Liu C, Towler M, Wyslouzil B. Mist reactors: Principles, comparison of various systems, and case studies. Electron J Integr Biosci 2008; 3: 29-37.

[46] Kim Y, Wyslouzil BE, Weathers PJ. A comparative study of mist and bubble column reactors in the *in vitro* production of artemisinin. Plant Cell Rep 2001; 20(5): 451-5.

[47] Sung L-S, Huang S-Y. Lateral root bridging as a strategy to enhanceL-DOPA production in *Stizolobium hassjoo* hairy root cultures by using a mesh hindrance mist trickling bioreactor. Biotechnol Bioeng 2006; 94(3): 441-47.

[48] Cham CL, Tan AH, Tan WH. Design and construction of a mist reactor system. IEEE Reg 10 Annu Int Conf Proceedings/ TENCON 2016: 3382-5.

[49] Dilorio AA, Cheetham RD, Weathers PJ. Reactor performance and evaluation. Appl Microbiol Biotechnol 1992; 37: 457-62.

[50] Sivakumar G, Liu C, Towler MJ, Weathers PJ. Biomass production of hairy roots of *Artemisia annua* and *Arachis hypogaea* in a scaled-up mist bioreactor. Biotechnol Bioeng 2010; 107(5): 802-13.

[51] Osama K, Pallavi S, Pandey AK, Mishra BN. Modelling of nutrient mist reactor for hairy root growth using artificial neural network. Eur J Sci Res 2013; 97(4): 516-26.

[52] Ranjan R, Ahmed N, Khanna R, Mishra BN. Design of an ON/OFF mist duty cycle in mist bioreactors for the growth of hairy roots. Biotechnol Bioprocess Eng 2009; 14(1): 38-45.

[53] Flores HE, Curtis WR. Approaches to understanding and manipulating the biosynthetic potential of plant roots. Ann NY Acad Sci 1992; 665: 188-209.

[54] Ramakrishnan D, Curtis WR. Fluid dynamic studies on plant root cultures for application to bioreactor design. In: Advances in Plant Biotechnology. Elsevier; 1994: 281-305.

[55] Manuhara YSW, Yachya A, Kristanti AN. Effect of aeration and inoculum density on biomass and saponin content of *Talinum paniculatum* Gaertn. hairy roots in balloon-type bubble bioreactor. J Pharm Biomed Sci 2012; 2(4): 47-52.

[56] Cardillo AB, Otalvaro AM, Busto VD, Talou JR, Velasquez LME, Giulietti AM. Scopolamine, anisodamine and hyoscyamine production by *Brugmansia candida* hairy root cultures in bioreactors. Process Biochem 2010; 45(9): 1577-81.

[57] Cui XH, Chakrabarty D, Lee EJ, Paek KY. Production of adventitious roots and secondary metabolites by *Hypericum perforatum* L. in a bioreactor. Bioresour Technol 2010; 101(12): 4708-16.

[58] Kochan E, Królicka A, Chmiel A. Growth and ginsenoside production in *Panaxquinquefolium* hairy roots cultivated in flasks and nutrient sprinkle bioreactor. Acta Physiol Plant 2012; 34:1-6.

[59] Mehrotra S, Kukreja AK, Khanuja SPS, Mishra BN. Genetic transformation studies and scale up of hairy root culture of *Glycyrrhiza glabra* in bioreactor. Electron J Biotechnol 2008; 11(2): 1-8.