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Nivedita Patra
Technological University Dublin

Ashok K. Srivastava
Indian Institute of Technology, Delhi, ashokiitd@hotmail.com

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Artemisinin production by plant hairy root cultures in gas- and liquid-phase bioreactors

Nivedita Patra[1,2] · Ashok K. Srivastava[1]

Abstract

Key message **Alternative biotechnological protocol for large-scale artemisinin production was established. It featured enhanced growth and artemisinin production by cultivation of hairy roots in nutrient mist bioreactor (NMB) coupled with novel cultivation strategies.**

Abstract Artemisinin is used for the treatment of cerebral malaria. Presently, its main source is from seasonal plant *Artemisia annua*. This study featured investigation of growth and artemisinin production by *A. annua* hairy roots (induced by *Agrobacterium rhizogenes*-mediated genetic transformation of explants) in three bioreactor configurations—bubble column reactor, NMB and modified NMB particularly to establish their suitability for commercial production. It was observed that cultivation of hairy roots in a non-stirred bubble column reactor exhibited a biomass accumulation of 5.68 g/l only while batch cultivation in a custom-made NMB exhibited a higher biomass concentration of 8.52 g/l but relatively lower artemisinin accumulation of 0.22 mg/g was observed in this reactor. A mixture of submerged liquid-phase growth (for 5 days) followed by gas-phase cultivation in nutrient mist reactor operation strategy (for next 15 days) was adopted for hairy root cultivation in this investigation. Reasonably, high (23.02 g/l) final dry weight along with the artemisinin accumulation (1.12 mg/g, equivalent to 25.78 mg/l artemisinin) was obtained in this bioreactor, which is the highest reported artemisinin yield in the gas-phase NMB cultivation.

Keywords Artemisinin · Hairy roots · Bubble column reactor · Nutrient mist bioreactor · Root support

Introduction

Artemisinin and its derivatives are highly effective drugs and given in combination to other drugs for the treatment of both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*, which causes cerebral malaria. Considering that more than 5 million people are infected with malaria every year worldwide, and its high dosage, the demand of artemisinin by society is extremely high which is not met by the plant-based artemisinin production alone. Therefore, there has been a growing need to find alternative economic production protocols to supplement the production of artemisinin (Basile et al. 1993). Mass production of artemisinin using plant cell/hairy root cultivation in bioreactor could be such a promising technique but there is a need to establish its growth and production ability in different bioreactor configurations, and optimize their operation strategy(ies) to scale up the production for commercial exploitation.

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Ashok K. Srivastava
ashokiitd@hotmail.com; ashokks@dbeb.iitd.ac.in

[1] Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Delhi 110016, India

[2] Present Address: School of Food Science and Environmental Health, College of Sciences and Health, Dublin Institute of Technology, Cathal Brugha Street, Dublin 1, Ireland

Stability of plant cells has been reported as a major bottleneck during plant cell cultivation; as a result, the biosynthetic capacity of cell for metabolite production invariably decreases with successive subculture cycles. Bioreactor hairy root cultivation, on the other hand, has been indicated as a promising technique primarily due to its genetic stability and its distinct ability to grow in hormone-free media. Hence, once obtained, hairy root culture can serve as a parent culture for continuous production of secondary metabolites (Sharp and Doran 1990). Owing to these advantages, its growth and production ability in different bioreactor configurations were examined primarily to investigate the suitability of the reactor, its operation strategy and possible scale-up for the mass economic commercial production.

A few reports are available in the literature, which focus on in vitro production of artemisinin in liquid-phase bioreactors using *Artemisia annua* hairy root culture (Kim et al. 2003; Liu et al. 1998, 2006; Patra and Srivastava 2015). However, it has been invariably observed that scale-up of hairy root cultivation from shake flask to large-scale bioreactors for industrial production of useful secondary metabolites has been a challenging task due to the complex morphology and non-homogeneous growth pattern of the hairy roots (Kim et al. 2013). The complex three-dimensional structure of hairy roots presents a major challenge during bioreactor cultivation. The dense matrix of hairy roots causes resistance to mass and oxygen transfer (Bordonaro and Curtis 2000). Local changes in environmental temperature and shear stress lead to significant changes in the morphology of roots. A slight change in morphology like root length, physiological condition of the roots or root thickness significantly affects the productivity of secondary metabolites (Bordonaro and Curtis 2000). Successful attempts were made in the past to mass cultivate the hairy roots of *A. annua* in modified 3-l Stirred tank reactor (1.5-l working volume) under optimum cultivation conditions. This reactor featured segregation of roots from the agitator of reactor by a perforated Teflon disc. This arrangement ensured adequate nutrient availability to growing roots without any mechanical shearing (Patra and Srivastava 2014a).

Modified bioreactor configurations like temporary immersion systems and hydroponics-based systems have been reported to be highly successful for mass propagation of plant tissues (Mujib et al. 2014). This bioreactor design ensures little or no alteration of the root morphology and also allows better nutrient and oxygen transfer into the core of the growing hairy roots. Mass transfer resistances in both solid–liquid and liquid–gas interface also adversely affects growth and secondary metabolite production during hairy root cultivation (Palavalli et al. 2012). Therefore, hairy root cultivation for secondary metabolite production relies heavily on the development of a suitable bioreactor configuration wherein several bioprocess parameters (mixing, oxygen transfer, shear force, etc.) have been appropriately accounted (Georgiev et al. 2013). The bioreactor design also has to take into account factors like hairy root support matrix, minimal mass transfer limitation, provision for light particularly for the production of light-dependent secondary metabolites.

Non-stirred type reactors like airlift reactors have been utilized for plant cell cultivation (Wen et al. 1996). Bioreactor cultivation of plant cells is reasonably complex and more or less similar to the cultivation of animal cells (Wu and Zhong 1999). Growth of plant cells and hairy root culture has been attempted to some extent and implemented in liquid-phase submerged reactors like stirred tank reactors (STR), convective flow reactor, bubble column reactor, airlift reactor, rotating drum bioreactor, centrifugal impeller bioreactor, spin filter bioreactor, etc. (Panda et al. 1989; Patra and Srivastava 2014a; Prakash and Srivastava 2007; Srivastava et al. 2008). Most of these bioreactor configurations were found to be suitable for enhancement of biomass growth. Also, gas-phase bioreactors were reported to be more suitable for obtaining high secondary metabolite concentration due to lesser hypoxic stress as well as high nutrient concentration availability arising out of evaporation of gases, particularly for hairy root cultures (Kim et al. 2001). However, for both growth and metabolite production from more differentiated hairy root cultures, which primarily grow as unorganized mass of tissue, gas-phase bioreactors (wherein the roots are exposed to nutrients in the form of mist or droplets) or a combination of gas-phase and liquid-phase configuration have been found to be much more appropriate. Examples of the latter category of reactors are nutrient spray bioreactor, trickle bed bioreactor, temporary immersion bioreactor, etc. (Towler et al. 2006; Towler and Weathers 2007; Weathers et al. 2010).

The main problem with scale-up of hairy roots for the mass production of secondary metabolites is the inadequate availability of the know-how regarding multi-gene regulated biosynthesis of secondary metabolites (Arsenault et al. 2010; Nguyen et al. 2011). Yet attempts should be made to translate the laboratory scale success stories to plant cell cultivation using the established protocols of scale-up of microbial cultivations.

Materials and methods

Induction of hairy roots

The induction of hairy roots was done according to the protocol reported in literature (Putalun et al. 2007) with

minor modifications as described earlier elsewhere (Patra et al. 2013). The hairy roots emerging from the cut ends of plant tissue were allowed to grow till it was about 4 cm in length. These were then aseptically excised from the parent plant and subcultured in MS (Murashige and Skoog) media for propagation of parent culture which was thereafter subcultured in MS media every 3 weeks for routine maintenance. Molecular characterization of hairy root line was thereafter done using PCR for *rol*A gene (root locus gene). The confirmation of transformed status of hairy roots was done according to a method standardized earlier for *Azadirachta indica* hairy roots (Srivastava and Srivastava 2012). The growth of hairy root culture and artemisinin accumulation was statistically optimized and reported earlier (Patra and Srivastava 2014b). The optimized media emerging from Plakett-burman design and Response surface methodology (RSM) were of the following composition: (NO_3/NH_4^+) ratio of 3.5, 0.5 mM KH_2PO_4, 37.13 g/l sucrose and 10 µg/l GA_3 (gibberellic acid) in (MS/4) medium (Patra et al. 2011).

Hairy root culture inoculum preparation, maintenance and inoculum transfer

The inoculum (1 g/l DW (dry weight)) used for hairy root cultivation in bioreactor was prepared by cultivating the hairy root culture in liquid MS medium for 8 days. This was initiated by the transfer of fresh hairy roots under sterile conditions in 500 ml erlenmeyer flasks containing 100 ml MS media. The flasks were kept in an incubator shaker rotating at 70 rpm for a period of 8 days under 16/8 L/D (light and dark) photoperiod regime. The temperature of the culture was maintained at 25 °C and the initial pH of the culture medium was set at 5.8. The hairy roots were subcultured in fresh MS medium after every 20 days of cultivation for regular maintenance of seed culture. The optimization of specific conditions of growth of hairy roots has been discussed in detail elsewhere (Patra and Srivastava 2014b). In brief, the optimized conditions for shake flask cultivation of *A. annua* hairy roots were agitation speed: 70 rpm, temperature: 25 °C, size of inoculum: 1.0 g/l on DW basis, age of inoculum: 8 days, medium volume to total shake flask volume (V_m/V_f) ratio: 0.18 ml/ml. Statistically optimized medium (nitrate/ammonium ratio: 3.5, KH_2PO_4: 0.5 mM, sucrose: 37.134 g/l, GA_3: 10 µg/l in MS/4 medium) was used for the cultivation of *A. annua* hairy roots in the bioreactor. Shake flask hairy root inoculum (1 g/l on DW basis) was later on aseptically transferred to the bioreactors. The pH and temperature of the cultivation broth were maintained at 5.7 ± 0.1 and 25 ± °C, respectively, using Biocontroller (Applikon Dependable Instruments, The Netherlands). The ambient air was enriched by mixing with 40 % v/v oxygen in the liquid-phase bioreactors to enhance the O_2 fraction in the air. Artemisinin production in plants has been reported to be light and oxygen dependent; therefore, provision for light was also done during the bioreactor cultivations.

Steps in hairy root cultivation in different bioreactor(s)

Batch cultivation of hairy roots was done in selected liquid- and gas-phase bioreactor configurations. Conventional bioreactors have adequate provisions for aeration and agitation, yet it adversely affects the growth of root tissues due to the highly sensitive nature of hairy roots. Moreover, hairy root growth is also severely affected by the immobilization of roots on various kinds of support structures like mesh, cage, hooks, foams, etc. Therefore, different supports for shear sensitive hairy roots were custom fabricated to ensure better biomass growth.

The bioreactors for plant cell/hairy root cultivation are complex primarily because these systems are shear sensitive and hairy root growth is also extremely slow, thereby, necessitating maintenance of perfect aseptic conditions for longer periods. All the bioreactor configurations used in the present study were, therefore, sterilized by autoclaving at 121 °C at 15 psi pressure for 20 min. The culture room housing the bioreactor was fumigated overnight before each bioreactor operation by mixing potassium permanganate and formaldehyde solution. The fumes, thus, generated were highly toxic and removed by forced air completely before the initiation of bioreactor operations. The culture medium was separately autoclaved in conical flasks and aseptically transferred to the bioreactor vessel in the laminar flow cabinet. During cultivation, air at a constant flow rate of 0.3 vvm was supplied to the bioreactor and dissolved oxygen (DO) level in the broth was monitored continuously. The temperature of the bioreactor was controlled at 25 ± 1 °C. The pH of the bioreactor medium was maintained at 5.7 ± 0.1 by the addition of 0.5 N hydrochloric acid or 0.5 N sodium hydroxide using the Biocontroller (ADI, The Netherlands) connected to the bioreactor. Samples were withdrawn at regular intervals of 2 days and analyzed for biomass, substrate and artemisinin concentrations.

Modified bubble column bioreactor

Batch cultivation of *A. annua* hairy roots was done in a 3-l bubble column bioreactor with floating perforated Teflon disc as root support. In this bioreactor configuration, air was sparged through the medium and the roots were completely submerged in the liquid medium. As agitation in this bioreactor has been through air bubbles, it features

less shear as compared to other mechanically agitated stirred tank reactors (STR) (Sánchez Pérez et al. 2006). The medium in the bubble column reactor was sterilized in the autoclave and after cooling the medium to desired temperature the reactor was connected to respective controllers. Cultivation was initiated by the aseptic transfer of hairy roots in pre-sterilized bioreactor. The bioreactor setup has been illustrated in Fig. 1a. Cultivation was done under 16/8 h light/dark conditions. At the end of the 20th day cultivation period, the reactor was harvested by complete removal of biomass from it and analyzed for DW, artemisinin content, residual sucrose, nitrate, phosphate, cell viability and fresh weight to DW ratio.

Nutrient mist bioreactor

The roots grown in liquid-phase reactors, like bubble column and airlift reactors, invariably experience hypoxic stress primarily due to inadequate aeration and/or less solubility of oxygen in the medium (Weathers et al. 1999). This problem was eliminated by growing the roots in gas-phase reactors like Mist reactor, which were sprinkled with nutrients of liquid droplets, mist or fog. In this investigation, a specialized nozzle was designed for generating nano-size nutrient mist particles (cloud) and was used in a 5-l bioreactor. The details of special nozzle have been shown and described in detail elsewhere earlier (Srivastava and Srivastava 2012). The roots were placed on a perforated stainless steel mesh placed on the top of the nozzle. The inoculum used for the bioreactor was 1 g/l (DW basis). The ambient temperature of the culture room was maintained at 25 ± 1 °C and the hairy root cultivation was done under 16/8 h light/dark condition. The medium flow rate from reservoir to nozzle was at a rate of 0.2 ml/min. The excess medium trickling at the bottom of the mist chamber was recycled back to the medium reservoir. Thus, even though the cultivation was in the batch mode, yet the roots were sprinkled with conditioned medium which promoted better growth of hairy roots as compared to fresh medium. The outlet air from the mist chamber was recycled back into the medium reservoir which was connected to the condenser to avoid change in the medium volume due to evaporation. The bioreactor setup has been shown in Fig. 1b. At the end of the 20th day cultivation cycle, the reactor was harvested by removal of biomass from it and analyzed for DW, artemisinin content, residual sucrose, nitrate, phosphate, cell viability and fresh weight to DW ratio.

Fig. 1 Hairy root cultivation in a modified bioreactors. **a** Modified bubble column bioreactor set-up (*T* teflon support, *S* sparger, *W* water supply to jacket of bioreactor from the chilling unit), **b** nutrient mist bioreactor (*Co* pH and temperature bio-controller, *M* medium reservoir, *P* peristaltic pump supplying nutrient medium to the nozzle). **c** Bubble column mode of modified nutrient mist reactor (*N* liquid nutrient medium, *Su* cotton support for hairy roots, *As* air sparger. **d** Mist mode of modified nutrient mist reactor (*Ms* mist cycle controller, *No* nozzle supplying nano-sized nutrient mist droplets, *L* three chokes to activate (complete) the electric circuit of three compact fluorescent lamps)

Modified nutrient mist bioreactor

The modified nutrient mist bioreactor (NMB) was operated initially as a normal liquid-phase reactor. In this phase, the hairy roots were completely soaked with nutrients. This avoided sudden shock upon transfer of roots from shake flask to nutrient mist cultivation conditions. Primarily, the hairy roots were first acclimatized to the liquid-phase reactor environment in the bubble column cultivation mode, wherein the roots were submerged in optimized medium for 5 days. The bubble column mode has been depicted in Fig. 1c. It was observed that the roots get attached to the support of the bio-reactor. Thereafter for the next 15 days, the nutrient was supplied to the growing hairy roots in the form of tiny nutrient mist droplets. A cotton support was used on the perforated stainless steel support to avoid contact of roots directly with metallic part of the culture chamber in the reactor. A schematic of the mist mode of cultivation is shown in Fig. 2 and picture of the setup is given in Fig. 1d.

The inoculum used for the reactor was 1 g/l (DW basis). The temperature of the culture room was maintained at 25 ± 1 °C. The cultivation was done under 16/8 h light/-dark conditions. At the end of the 20th day cultivation, the reactor was harvested by removal of biomass from it and analyzed for DW, artemisinin content, residual sucrose, nitrate, phosphate, cell viability and fresh weight to dry weight ratio. The bioreactor cultivation was repeated 3 times to ensure reproducibility. The data shown in this report reflect the average of process variables obtained from 3 independent bioreactor experiments. The growth rate was calculated as residual biomass (final–initial) per unit initial biomass per day.

Analytical methods

Medium from the bioreactor was collected under sterile conditions after every 2nd day for the analysis of sucrose, nitrate and phosphate. Hairy root biomass was, however, estimated on DW basis only at the end of the cultivation. Essentially, the hairy root bed (mass) was separated from the bioreactor broth. It was then rinsed with deionized water once and blotted dry using blotting paper. The root bed was then weighed for the estimation of fresh weight. DW was estimated when the root bed exhibited no change in mass after drying it for 7 day at 30 °C.

Total sugar in the broth was estimated using a phenol sulfuric acid method (Dubois et al. 1956). Nitrate was also estimated by colorimetric method (Cataldo et al. 1975). Total nitrate concentration was established by comparison with a standard curve of nitrate vs OD at a wavelength of 410 nm.

The viability of the hairy roots was established by the tetrazoliumtrichloride dye (TTC) assay (Brunner et al. 2002). Phosphate concentration in the supernatant was measured by a colorimetric assay based on the formation of a blue color complex with molybdate ions (Murphy and Riley 1962). Sucrose was completely hydrolyzed by adding an equal volume of hydrochloric acid (1 M) and boiling the resulting mixture at 100 °C for 15 min. The reducing sugars so produced were estimated by di-nitro salicylic acid (DNSA) colorimetric method (Miller 1959). Residual sucrose was estimated by the phenol sulfuric acid method as well as DNSA method and the results were compared to ensure accuracy.

Hairy roots were separated at the end of the cultivation and used for the estimation of total artemisinin content. Extraction of artemisinin was done according to a method

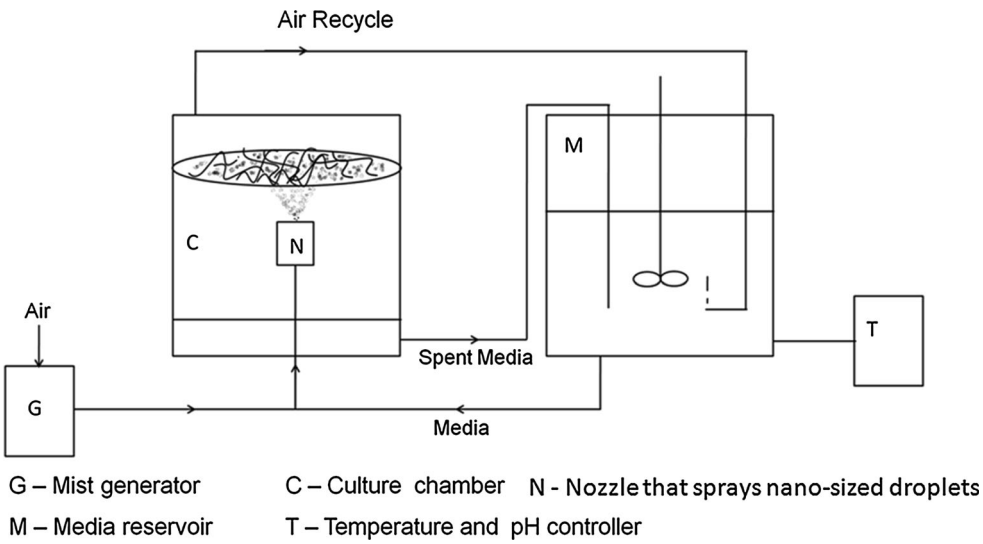

Fig. 2 Schematic diagram of nutrient mist reactor

reported in literature (Smith et al. 1997). The separation of sodium derivative of artemisinin was done using Agilent 1200 series HPLC (High–performance liquid chromatography) system, by a reversed-phase C18 analytical column using the mobile phase as 0.01 M sodium phosphate buffer and methanol (in 1:1 ratio). The pH of the buffer was adjusted to 7.0. The flow rate of HPLC solvent system was set at 1 ml/min. The retention time for artemisinin was in the range of 5.4–5.8 min. Crystalline artemisinin was obtained from Sigma (St. Louis, USA) and was used as standard for the estimation of artemisinin concentration and peak time validation.

Results

Bubble column reactor has been one of the most preferred reactors for mass scale propagation of various hairy root cultures. It is shown in Table 1 that the fresh weight obtained in this bioreactor configuration was 37.5 ± 0.02 g/l (5.68 ± 0.02 g/l DW) and artemisinin concentration was 1.50 mg/l after 20 days of cultivation. It was observed that adequate nutrient (28.2 g/l residual sucrose) and oxygen (dissolved oxygen concentration was more than 112 % of ambient saturation concentration due to bubbling of 40 % v/v pure oxygen with air) were available for the growth of hairy roots; however, the growth was not homogeneously distributed across the reactor. The biomass yield and artemisinin productivity were observed to be poor in this bioreactor configuration, possibly due to non-homogeneous growth of hairy roots. The hairy roots were floating (not immobilized) in this bioreactor configuration due to aeration and mixing. This led to shear stress to the hairy roots throughout the cultivation.

The roots grown in liquid-phase reactors like Bubble column and Airlift reactor feature hypoxic stress due to poor aeration and less solubility of oxygen in the culture medium as compared to gas-phase reactors. This was due to the fact that the saturation concentration of DO (dissolved oxygen) in water is generally as low as 8 ppm (which decreases for culture medium) as compared to air which features higher (21 %) oxygen availability. This problem was overcome by growing hairy roots in gas-phase reactors like Mist reactor. In nutrient mist bioreactor, the nutrient was sprayed in the form of fine nano-sized droplets (Srivastava and Srivastava 2012; Wyslouzil et al. 2000). The reactor consists of a mist generator (ultrasonic, acoustic or nozzle type), nutrient reservoir and a culture chamber. The bioreactor experimental setup is shown in Fig. 1b. The mist generation system was controlled by an independently designed timer, wherein the optimum mist cycle was found to be 5 s 'on' and 30 s 'off'. Table 1 summarizes the biomass, artemisinin yield and productivity in a conventional nutrient mist reactor. The biomass dry weight obtained in this bioreactor configuration was 8.52 ± 0.93 g/l, but reasonably, low artemisinin production 2.14 mg/l (and artemisinin content of 0.25 mg/g) was observed.

To overcome the major limitation of normal mist bioreactor cultivation, suitable modifications were introduced in the design and operation of the bioreactor. As seen in Table 1, the final dry weight obtained in this bioreactor configuration was 23.02 ± 2.61 g/l. The higher growth of biomass (23.02 ± 2.61 g/l on DW basis after 20 d) and hence more volumetric productivity of artemisinin (1.29 mg/l days) in the bioreactor could be presumably due to more nutrient availability during gas phase due to better retention of nutrients partly by the cotton support during the initial liquid-phase reactor mode and no abrupt shock onto the roots on transfer from shake flask liquid medium to nutrient mist environment as the roots were first acclimatized and soaked in medium in the bubble column reactor. In this cultivation mode, the roots were provided with optimized medium for initial 5 days in which the roots got firmly attached to the cotton root support. In the next 15 days, nutrient was supplied to the roots only in the form of nutrient mist. The two different cultivation modes have been depicted in Fig. 1c, d. In the modified NMB, a dense layer of hairy roots was formed on the mesh after 20 days. The better growth of hairy roots in combination reactor may be due to absorption of liquid medium by the cotton support on mesh during liquid phase, thereby ensuring better nutrient availability throughout the cultivation period. The cotton support could not absorb and retain nutrient medium in a conventional NMB where nutrient was only sprayed in the form of mist. A previous aerosol model for growth of hairy roots in a mist reactor also indicated that

Table 1 Summary of bioreactor cultivation results

S. no	Type of cultivation	Biomass (g/l)	Growth rate (day^{-1})	Artemisinin (mg/g)	Productivity mg/(l d)
1	Modified BCR (3–l)	5.68 ± 0.02	0.17	0.27 ± 0.001	0.06
2	NMB (5–l)	8.52 ± 0.93	0.10	0.22 ± 0.030	0.07
3	Modified NMB (5–l)	23.02 ± 2.61	0.45	1.12 ± 0.140	1.29

Values are mean ± standard deviation of the average (mean) values

effective absorption of mist is highly important for growth (Towler et al. 2007). In this investigation also, the effective absorption of mist was enhanced using the moist cotton support. The medium reservoir temperature and pH were strictly controlled at optimum cultivation conditions using a Bio-controller (ADI, The Netherlands). As a result, 25.78 mg/l (1.12 mg/g) of artemisinin and 23.02 g/l biomass were obtained after 20 days of cultivation.

Table 1 summarizes a comparison of bioreactor cultivation results of this investigation. It can be concluded from Table 1 that the cultivation of *A. annua* hairy roots in modified nutrient mist bioreactor (5–l scale) resulted in highest biomass accumulation of 23.02 g/l (highest literature reported so far in batch cultivation in a mist bioreactor) with artemisinin content of 25.78 mg/l (1.12 mg/g). The summary of substrate utilization in the modified nutrient mist reactor batch cultivation has been shown in Table 2. However, there was no statistically significant difference in terms of biomass growth yield in gas- and liquid-phase bioreactors. The biomass growth yield per unit limiting substrate was found to be 0.67 g/g of sucrose consumed in modified NMB, as compared to biomass yield of 0.64 g/g sucrose consumed in modified stirred tank bioreactor and 0.65 g/g in shake flask cultivation (Patra and Srivastava 2014a). Initial phosphate concentration in the medium has little effect on artemisinin content and nitrate concentration remains non-limiting till stationary phase in *A. annua* hairy root cultivation (Patra and Srivastava 2015). Some literature reports have described apparent biomass growth yield per unit limiting nutrient (sucrose) to be 0.35 g/g glucose equivalent for *A. annua* hairy root cultivation in a nutrient mist reactor. However, the total sugar concentration in terms of glucose equivalent per litre was obtained by the sum of glucose, sucrose and fructose concentration in the literature reported results (Kim et al. 2002a, b). This apparent biomass yield in previous reports is much lower than the biomass yield of the present investigation.

Discussion

In Bubble column reactor cultivation, less biomass and artemisinin content (Table 1) were obtained primarily because the hairy root distribution was not uniform and hairy roots tend to form an entangled mass which accumulated near the sparger inlet due to inadequate mixing. Probably, better distribution of the hairy root cultures in the bioreactor could be achieved by increasing the number of possible active growth nodes by providing a draft tube support wherein the hairy roots could be entangled through a wire mesh (Kwok and Doran 1995). The mesh could function as a support for the growth of hairy roots and could provide hairy root attachment sites. Specialized absorbent polyurethane foam mesh has also been reported to show better growth of hairy roots as compared to conventional stirred tank reactor and bubble column reactors (Srivastava and Srivastava 2013). Fed-batch cultivation has also been reported to be a promising cultivation strategy to enhance the production of secondary metabolites like betalains in bubble column reactor (Pavlov et al. 2007).

It has been reported by other investigators also that the hairy roots grown in shake flask and liquid-phase reactors like bubble column experience serious oxygen deficiency (Kim et al. 2001, 2002a, b; Weathers et al. 1999). Hairy root cultivation of *Hyocyamus muticus* in a 15 l bubble column reactor has been used for the production of plant secondary metabolites (Bordonaro and Curtis 2000). Bubble column reactor has also been reported for the production of taxol from *Taxus cuspidate* cell culture (Son et al. 2000). The effect of low oxygen supply was studied in *Arabidopsis thaliana* hairy roots grown in a bubble column reactor (Shiao et al. 2002) which featured excellent heat and mass transfer characteristics. The reactor design was simple, compact and inexpensive, particularly with respect to energy requirement due to lack of moving parts (Li and Prakash 1999, 2001). In a bubble column reactor, the volumetric mass transfer coefficient (K_La) and heat transfer coefficient may also increase by increasing the gas velocity, gas density and gas pressure and decrease by increasing the liquid medium viscosity. Mass transfer has been reported to be higher in the center of the bubble column as compared to that near the walls due to the presence of a larger number of bubbles in the center. Higher gas holdup (indicating the volume of gas phase occupied by gas bubbles in the liquid phase) is also reported to be crucial for better design of bubble column reactor (Bouaifi et al. 2001; Luo et al. 1999; Terrier et al. 2007).

A nutrient mist reactor is easier to scale up due to the availability of nutrients in the mist form. The growth of

Table 2 Summary of substrate utilization data in modified nutrient mist reactor batch cultivation studies of hairy roots of *Artemisia annua*

S. no.	Variable	Sucrose (g/l)	Nitrate (mM)	Phosphate (mg/l)	Cell viability (%)
1	Initial value	37.5	38	68	100
2	Utilized (consumed) value	34.1	26	68	73
3	Biomass yield per unit limiting nutrient (g/g sucrose consumed)	0.67	–	–	–

hairy roots has been reported to be much higher and faster in nutrient mist bioreactor as compared to the shake flask and other liquid-phase cultures due to the exposure to nutrients in the form of mist. In liquid-phase cultures, roots form dense clumps and intra-clump fluid velocity has been reduced. Till date, the largest bioreactor reported for nutrient mist reactor was of 500-l capacity and was used for hairy root culture of *Datura stramonium* which gave 1 % yield of hyoscyamine (Wilson et al. 1990). This reactor could also be operated in submerged cultivation mode. Nutrients can also be supplied in the form of liquid droplets. This method has been normally used in a trickle bed bioreactor (Grzegorczyk and Wysokinska 2010). The various mist bioreactor configurations used earlier for *A. annua* hairy roots were acoustic window mist reactor (Souret et al. 2003; Towler et al. 2006, 2007), submerged ultrasonic bioreactor (Dilorio et al. 1992; Liu et al. 1999) and sonic nozzle in mist reactor (Liu et al. 2009). The less biomass and artemisinin concentration obtained in a conventional nutrient mist (Table 1) could be attributed to starvation of hairy roots due to an inadequate supply of nutrients through mist. Efficient supply of nutrients can be ensured by increasing the misting cycle which increases the medium flow rate (Liu et al. 1999; Towler et al. 2006). However, increasing the misting cycle beyond a certain limit also affects growth negatively as mass transfer resistant thin-film deposits over the root surface during 'mist duty ON' period (Towler et al. 2007). Other factors that affect growth of roots in a nutrient mist bioreactor are mist flow direction, inoculum distribution, gas composition, medium concentration (Chatterjee et al. 1997; Wyslouzil et al. 1997, 2000), etc. It has been advocated that conditioned medium has been much better than fresh medium for growth of hairy roots in mist bioreactor cultivation (Wyslouzil et al. 2000) as roots exude useful chemicals like oligosaccharides, auxins and peptides. Various modifications of nutrient mist reactors have been reported for mass scale hairy root cultivation (Buer et al. 1996; Chatterjee et al. 1997; Liu et al. 1999). In most of these bioreactors, separate reservoir and culture chamber lead to complications in the operation of the reactor like poor growth due to loss of medium due to evaporation, need to collect the mist, etc. Therefore, more advanced nutrient mist reactors in which mist generator and culture chamber are unified emerged for better operation (Liu et al. 1999; Woo et al. 1996).

From the data summarized in Table 2, it was observed that in a modified nutrient mist bioreactor the biomass yield (0.67) was much better than the yield reported earlier in modified stirred tank bioreactor (0.64) (Patra and Srivastava 2014a). The biomass (23.02 $\pm$ 2.61 g/l) and artemisinin concentrations of 25.78 mg/l (1.12 mg/g DW) reported in this investigation are significantly higher than the literature results for batch cultivation of hairy root cultivation in bioreactors. The production of artemisinin in liquid-phase reactor is indicated to be growth associated (Patra and Srivastava 2014a). Liquid-phase reactors have been reported to promote higher biomass concentration (Kim et al. 2001). Therefore, provision of an initial liquid-phase environment led to a significant enhancement of growth-associated artemisinin production in modified nutrient mist reactor (1.12 mg/g) as compared to conventional nutrient mist reactor (0.22 mg/g DW). By introducing an initial growth period of hairy roots in liquid phase (5 days), the overall hairy root growth and viability improved significantly in the modified nutrient mist bioreactor. This modification also reduced necrosis and browning of hairy roots (decay) due to the shorter mist mode in the modified nutrient mist reactor (15 days) as compared to the prolonged mist mode (20 days) in the conventional nutrient mist reactor. The root viability was less in conventional NMB presumably due to limited nutrient availability, which further decreased after 15 days of cultivation. Table 3 summarizes the results of artemisinin

Table 3 Bio-reactors used for artemisinin production using hairy root culture

S. no.	Type	Artemisinin reported	Literature reports
1	Nutrient mist bioreactor (1.8-L)	0.07–0.29 μg g^{-1} DW	Kim et al. (2001)
2	Nutrient mist bioreactor (2-l)	Artemisinin not reported	Liu et al. (1999)
3	Modified bubble column reactor (3-l)	0.025 mg l^{-1}	Kim et al. (2003)
4	Modified nutrient mist bioreactor (1.8-l)	0.031 mg l^{-1}	Kim et al. (2003)
5	Disposable culture bag mist reactor (20-l)	Artemisinin not reported	Sivakumar et al. (2010)
6	Modified stirred tank bioreactor (STR) (3-l)	4.63 mg l^{-1}	Patra and Srivastava (2014a)
7	Modified STR with elicitor	10.33 mg l^{-1}	Patra and Srivastava (2014a)
8	Model based fed-batch cultivation in modified STR	13.68 mg l^{-1}	Patra and Srivastava (2015)
9	Modified bubble column reactor (3-l)	1.50 mg l^{-1}	Present study
10	Nutrient mist reactor (5-l)	1.87 mg l^{-1}	Present study
11	Combination bioreactor (modified NMB) (5-l)	25.78 mg l^{-1}	Present study

Fig. 3 Hairy root morphology in different bioreactors: **a** liquid-phase bioreactor, **b** combination of gas- and liquid-phase bioreactor (modified NMB)

accumulation obtained using batch cultivation in nutrient mist bioreactor and other bioreactor configurations. Figure 3 shows the images of plant roots grown under liquid-phase and modified nutrient mist bioreactors. In the modified nutrient mist bioreactor, the root tips were found to be fast growing, highly branched, plagiotropic and hairy in appearance. The hairy appearance of roots in the mist mode of bioreactor operation helps them to capture and retain the mist more efficiently and also provides more surface area for gaseous exchange. In contrast, the roots grown in a liquid-phase reactor are less hairy, slow growing and comparatively less branched. Such a modified nutrient mist bioreactor is, therefore, much better than the conventional mist bioreactor and not only has ultimate commercial application for artemisinin production using hairy root cultivation but also can be useful as a model nutrient mist bioreactor configuration for other systems.

Therefore, a combination of bubble column and nutrient mist bioreactor was found to be most suitable for high biomass production and large-scale artemisinin production in a bioreactor using hairy root cultivation.

Conclusion

The hairy root culture of *A. annua* was grown in three bioreactor configurations to assess their suitability for economic and commercial production—bubble column reactor, nutrient mist reactor and modified nutrient mist bioreactor. Mass cultivation in non-stirred bubble column reactor for the cultivation of shear sensitive *A. annua* hairy roots resulted in biomass accumulation of 5.68 g/l. The hydrodynamic stress to the hairy roots during batch cultivation was significantly minimized, while the other cultivation conditions were optimized, e.g., mist duty cycle (5 s on 30 s off), root support (cotton), medium flow rate (0.2 ml/min), light conditions (fluorescent white light), efficient inoculum distribution, gas composition (ambient air mixed with 40 % v/v oxygen), medium concentration (statistically optimized and conditioned medium) and the use of a custom-made nutrient mist bioreactor batch cultivation, which featured still higher biomass concentration of 8.52 g/l but low artemisinin accumulation of 0.22 mg/g. Appropriate modifications were implemented in the nutrient mist bioreactor to facilitate enhanced artemisinin accumulation. In the modified nutrient mist bioreactor, the roots were first submerged in the bubble column reactor mode for the initial 5 days of cultivation followed by nutrient supply by mist mode for the next 15 days of growth. The final dry weight obtained in this bioreactor configuration was 23.02 g/l and the artemisinin accumulation was 1.12 mg/g (equivalent to 25.78 mg/l artemisinin) which is the highest reported artemisinin yield in a gas-phase nutrient mist bioreactor so far.

Author contribution statement This is to confirm that Dr. Nivedita Patra was my Ph.D. student. The experimental part of the research work reported in the manuscript has been done entirely by her. I have been her thesis supervisor to guide her research work.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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