Journal of Innovative Agriculture: 8(1) : 1-10, 2021
DOI: 10.37446/jinagri/ra/8.1.2021.1-10
Review paper

Hairy root culture: a promising alternative for enhancing the production of biologically active compounds

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Edited by
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Received: 24 September 2020
Accepted:16 February 2021
Published: 31 March 2021

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Hairy roots are obtained from the infection caused by *Agrobacterium rhizogenes, a* gram negative bacterium and are known to produce different complex molecules. Various biochemical pathways and physiological aspects in plants can be understood by means of hairy roots. Being genetically and biosynthetically stable as well as resultant high biomass accumulation and productivity in short period of time, these roots are great alternatives to conventional methods for the production of pharmacologically important compounds. Various biotechnological approaches i.e. culture medium components and their concentration, culture conditions, elicitation etc. are used and optimized to enhance overall yield. To meet up the increasing demand, production on industrial scale has been considered to be an important where concept of bioreactors is involved. This review presents basic idea of development of hairy roots, requirement of the optimum culture conditions and use of bioreactors to increase yield of the bioactive compounds.

Key words: hairy roots, Agrobacterium rhizogenes, metabolites production, bioreactor cultivation

INTRODUCTION

Secondary metabolites or the bioactive compounds formed as a result of secondary metabolism in plants are destined to perform specific functions. Humans have been using these metabolites for a long period of time to satisfy various needs (Cragg and Newman, 2013). Since the plants growing in their natural habitat are an important source of medicinally important compounds required by the pharmaceutical industry for the drug development, the varied environmental factors greatly influenced the metabolite profiling of the plant. Moreover, the structural complexity and chirality of these target secondary metabolites make it almost impossible to chemically synthesize, even if it happens that is not economically feasible due to high cost of its production (Almagro et al., 2013). Since these bioactive compounds are produced by plants in order to interact with their environment, this property directed scientists towards the modern approach that not only is natural but is environment friendly and highly stable. This approach is production via plant tissue culture techniques. Initially cell suspension culture remained popular for this purpose and is still in use. Recently, hairy root culture has attracted widespread attention as a more stable and prominent method in this field. These genetically transformed roots cultivated under artificial conditions can produce highly diverse molecules.

The production of hairy roots depend on many factors such as capability of plant to produce particular secondary metabolite, complexity of that molecule, whether it is toxic or not, its molecular weight etc. While selecting most appropriate plant species, the ability of the plant to produce high amount of molecule of interest and total biomass production are two important features that need to be taken in consideration.

1. Hairy Root Culture: An Attractive Alternative

Hairy root culture proved to be an attractive technique for the production of secondary metabolites in comparison to the parent plant from which they are extracted. This property along with being genetically and biochemically stable plus rapid growth in basal media and possible extracellular secretion of expressed proteins (rhizosecretion) makes them attractive alternative to conventional methods. Hairy roots drew attention as perfect resources for chemical compounds that are of utmost importance, or certain metabolites that are crucial and are present in medicinal plants that are either under threat or at the verge of extinction. A laboratory protocol was provided for the initiation, genetic transformation and culturing of these roots (Doran, 1997).

Entire process begins with induction of hairy roots by *A. rhizogenes* which has been defined in sequential manner (Figure 1). Generally bacterial inoculum is used to infect the wounded explants. If the production is considered with recombinant proteins, genetic manipulation in bacterial plasmid DNA is done for the incorporation of desired genes which can further be expressed in hairy roots. Explants are further subjected with antibiotics to remove bacteria. As a result hairy roots can be seen growing in hormone free culture media. PCR is performed in order to confirm that these roots are not adventitious. PCR uses primers that amplify *rol* and *vir* genes. After the selection and development of high yield producing HR strain, its preservation becomes important. Monthly subculture of HR's on solid/liquid media is currently in use. However being time consuming and expensive process, it has high risk of contamination. Georgiev et al. (2012) attempted the cryopreservation procedure for the preservation of important hairy root clones.

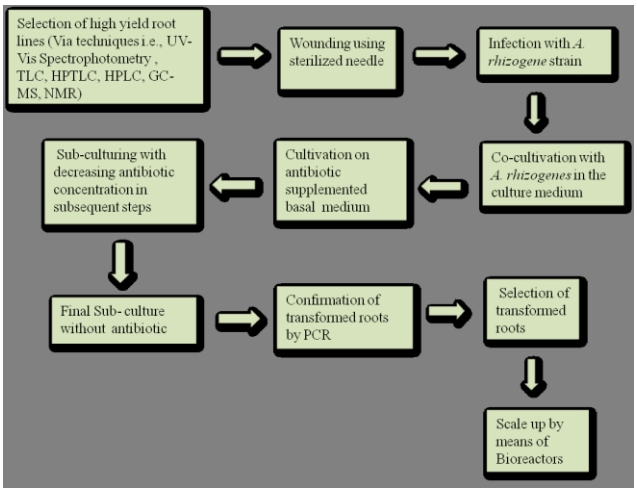

Figure 1. General protocol followed for the production of hairy roots

Once the culture established, its production on large scale can be achieved. Bioreactors are ideal resource to increase the overall production. These are controlled systems that provide the necessary environment to hairy roots for growth and proliferation and are monitored throughout the process. But large scale production itself is challenging because of the complexity of the process as thorough knowledge is required. Investigations on several optimization steps led to the advancements in design and construction of a bioreactor which resulted in the production of several secondary metabolites in few plant species (Patra and Srivastava, 2014a,b, 2015, 2017). There are many factors which need to be considered in order to produce desired secondary metabolites as hairy roots are very delicate in nature hence their scale up via bioreactors can be challenging. Hairiness, thickness, length and the branching of the hairy roots are few factors that need consideration (Thakore *et al*., 2017).

To sum this up, many biotechnological approaches have been considered and further developed in order to investigate their overall effectiveness towards the improvement in growth rate and total productivity of the bioactive compounds. This includes selection of the desired root line capable of high secondary metabolite production; optimization of the culture medium and the conditions of culture such as selection of suitable medium, pH, temperature, aeration, light quality, illumination, agitation, and gas (O_2 and CO_2); replenishment of the nutrient and the precursor feeding (Zhu *et al*., 2013; Srivastava and Srivastava, 2014); elicitation (Wang and Wu, 2013; Belabbassi et al., 2016); phytohormonal application and metabolic engineering (Golkany et al., 2013; Zhao et al., 2013; Sun and Peebles, 2016); and scale-up via bioreactors (Dehghan et al., 2012; Murthy et al., 2014).

1.2. Agrobacterium rhizogenes: a potential transformation tool

Plant infection caused by bacterium *Agrobacterium rhizogenes* (also known as *Rhizobium rhizogenes*) results in the formation of hairy roots. This gram negative bacterium contains Root inducing (Ri) plasmid or vector that is ultimately responsible for the entire transformation and resultant hairy roots. Ri plasmid contains a fragment of DNA named T-DNA that gets randomly incorporated in host plant's genome. Apart from T-DNA there are other loci that plays crucial role in transformation process namely *vir* region of pRi and chromosomal virulent (*chv*) genes . Genes *vir* D1 and D2 form certain proteins which particularly forms cut at 25 bp T-DNA border repeat sequences in DNA (Georgiev et al, 2012). The other *vir* genes namely *vir* E1 and *vir* E2 are also significant as they protect T-DNA from digestion by nucleases and ease the process of integration into host plant genome by pRi GALLAS genes which portray certain proteins for the helicase activity and nuclear localization signal (Gelvin, 2009). The left and right DNA are two independent sequences present in T-DNA namely TL and TR respectively. This DNA induces the formation of hairy roots. TL DNA consists of root origin locus or *rol* genes i.e., *rol* A, *rol* B, *rol* C and *rol* D. Studies have shown that all the four *rol* genes have specific functions however *rol* B is more involved in hairy root induction followed by *rol* C. Both *rol* C and *rol* B are also involved in activation of phosphorylation / dephosphorylation processes. These processes play key role in signal transduction pathway which is involved in molecular recognition of elicitors and activation of plant defence reactions (Bulgakov *et al*., 2002; Chandra, 2012)

2. Factors affecting the growth and biomass accumulation

Throughout years, various experimentations conducted in this particular field suggests that different biotechnological approaches like selection of a desirable root line, kind of the basal medium suitable for growth as well as the concentration of different nutrients present in basal medium, conditions of the culture medium including density of the bacterial inoculum, temperature and light, use of elicitors can affect the productivity as described below:

2.1. Selection of desired root line

As far as the process of the secondary metabolite's production *in vitro* is concerned, there are three basic factors that greatly influence the growth and development of hairy roots. They are the genotype of the parent plant, the constituents of the culture medium in which they are supposed to be grown and the surrounding environment. Particular root line is selected by analyzing its growth rate following quantification process of the desired product by UV visible spectrophotometry and advanced chromatography techniques. The hairy root lines differ from each other on the basis of the copy number, total length and the position of the T-DNA integrated in host genome. The integrated T-DNA can create variability in growth rate, morphology and ability to accumulate desired phytochemicals (Halder and Jha, 2016). Proper screening and selection of root lines among the induced root lines has influenced total secondary metabolite production in *Plumbago zeylanica* for the production of plumbagin in hairy root culture (Nayak et al., 2015). Similarly in *Astralagus membranaceus* for the production of isoflavanoids (Jiao *et al.*, 2014) and total astragalosides (TAG) (Jiao et al., 2015); *Isatis inctoria* for the production of flavonoids and total alkaloids (Gai et al., 2015 a,b).

2.2. Optimization of culture medium

There are several factors including both physical and chemical such as basal medium, nutrients, growth regulators that affects total productivity (biomass and secondary metabolites) in plant cell and organ cultures (Murthy et al., 2014). These factors can be easily manipulated and have great impact on the production of the secondary metabolites. Hence optimization of the culture media is necessary.

2.2.1. Nutrients in culture medium and Salt strength

Macro and micro nutrients in the basal medium, physical status (liquid or solid) of the medium and salt strength influence the productivity of secondary metabolites with respect to the species or genotype of the plant (Carlin et al., 2015; Chung et al., 2016). Several studies have reported MS medium to be suitable for growth and production of desired secondary metabolite (Table 1). Thiruvengadam *et al.* (2016) cultured hairy roots of *Momordica dioica* on full strength MS medium supplemented with 3% sucrose resulted in optimum accumulation of biomass as compared to different media tested. Further, a significant increase in phenolic and flavonoid content was observed in transformed roots (Thriuvengadam et al., 2016). Saravanakumar et al. (2012) demonstrated that MS liquid medium of half- strength was more efficient for biomass accumulation and withaferin A production as compared to MS medium of full strength and B5 medium in hairy roots of *Withania somnifera*. In *Artemisia vulgaris* hairy root culture, half strength MS medium supported higher biomass production and among identified compounds camphor and camphene (Sujatha et al., 2013). Higher anthraquinone constituents were accumulated in hairy roots of *Polygonum multiforum* when supplemented with MS medium (Full strength) as compared to B5, white media (Huang et al., 2014). In *Rubia tinctorium* hairy root cultures supplemented with half strength B5 medium and Woody Plant Medium (WPM), increased growth rate and anthraquinone production was reported (Perassolo et al., 2017).

2.2.2. Carbon source and concentration

The growth rate and ability to accumulate secondary metabolites is greatly influenced by source of carbon and its concentration. Literature shows the effects on the growth and accumulation of complex chemical compounds because of the usage of various carbon sources as well the concentration in hairy root cultures (Shinde et al., 2010). Generally a single simple sugar or combination of the simple sugars i.e., glucose, fructose, maltose and sucrose can be the carbon source (Murthy et al., 2014). In hairy root culture of *Astragalus membranaceus*, sucrose was used as carbon source where 3% sucrose concentration in culture medium favoured optimum astragaloside accumulation, 4% concentration favoured biomass accumulation (Jiao et al., 2015). In hairy root culture of *Gentiana dinarica* the overall increase in sugar concentration resulted in negative impact on the phenolic production. Content of xanthone increased when medium is supplemented with sucrose but highest xanthone accumulation was reported when culture medium was supplemented with glucose or fructose. (Vinterhalter et al., 2015)

2.2.3. Concentration of phosphate

Inorganic phosphate is one of the crucial macronutrient to plant since it is a constituent of many functional molecules that are involved in various physiological processes (Peret et al., 2011). Phosphate is obtained by plants from soil in soluble form. The concentration of this element is extremely crucial. Optimum range can greatly benefit plant. Since topic of consideration is hairy root culture, this element is fed to growing culture through culture media. Low concentration of phosphate can affect as well as inhibit the growth hence various studies were performed to determine the optimum range of concentration in order to maintain the growth and yield. In a case study, Shinde et al. (2010) proposed that the lower concentration of phosphate favoured isoflavones production in *Psoralea corylifolia* hairy root cultures. In *Salvia miltiorrhiza* hairy root culture, the production of phenolic acid increased when culture media was deprived of phosphate which in turn induced key enzyme genes to run positive feedback pathway that affected tyrosine derived pathway more than the pathway involving phenylalanine (Liu et al., 2016).

2.2.4. Source of nitrogen and the ratio of NH_4^+/NO_3^-

Nitrogen is present as largest constituent of atmospheric air, plays crucial role in growth and development of plants. Since nitrogen can't be taken directly in gaseous form, it is rather fixed in the form of nitrate and ammonium. These compounds hence are added to various culture media as nitrogen sources with respect to the need of the hairy roots developing from the parent plant. The ammonium (NH_4^+) to nitrate (NO_3^-) ratio of the medium affected the productivity in hairy root cultures of some plant species (Shinde et al., 2010; Liu et al., 2013; Sharafi et al., 2013; Murthy et al., 2014). High biomass yield and maximum alkaloid production In *Anisodus acutangulus* hairy root cultures was achieved in 90 mM nitrogen concentration with 4:1 ratio of NH_4^+/NO_3^- , whereas nitrogen concentration lower or higher to this ratio showed inhibitory effects on both alkaloid as well as biomass production (Liu et al. 2013). However, Sharafi et al. (2013) suggested that in hairy root cultures, NH_4^+/ NO_3^- ratio of 20:10 mM favoured the accumulation of biomass in liquid MS medium. For optimum growth and biomass accumulation in *Turbinicarpus pseudopectinatus* cultures, Heller medium (low nitrogen medium) was favourable (Carlin et al., 2015). However, more systematic studies are required to understand the actual mechanism of enhancement and expression of enzymes that are involved in a particular metabolic pathway.

2.2.5. Phytohormones

Transformed hairy roots have the property of growing on phytohormone free basal media since auxin synthesizing genes are present in T-DNA incorporated in host genome. Non transformed roots need external supply of auxins. However the effect of auxins has been reported in some case studies. In *Hyoscyamus albus* culture, when media was supplemented with external kinetin and Indole-3-acetic acid (IAA), the total alkaloid content increased (Sauerwein *et al.,* 1992). In *Plumbago zeylanica*, selected hairy root lines had been subjected to auxins at different concentrations in optimized basal media, the exogenous application of auxin inhibited the growth and hairy root formation (Nayak et al., 2015).

2.3. Enhancement by means of elicitors

Elicitors are extracellular signal molecules released by plant cells to initiate plant defense responses and phytoalexin synthesis (Eder and Cosio, 1994). This definition excludes the abiotic factors that enhance the secondary metabolite production. Elicitors let plant to perceive that it is undergoing pathogenic attack or environmental stress. Under this perception plants can initiate number of signal transduction pathways that increase the expression of plant defense genes. The elicitors can be classified into abiotic (minerals, physical and chemical) and biotic (are from plant or pathogenic origin). Elicitation is used when cultures reach stationary phase at about 2-3 weeks from inoculation (Namdeo, 2007).

Abiotic elicitors such as Sodium acetate (10.2 mM) addition to *Arachis hypogaea* hairy root cultures for 24 h led to the secretion of resveratrol and trans-reveratrol upto 60 folds (Medina- Bolivar et al., 2007). Heavy metals, for example cadmium (Cd), stimulated the production of ajmalicine in *Catharanthus roseus* hairy root cultures hence proven to be a potent elicitor (Zheng and Wu, 2004). Use of Silver (Ag) escalated Atropine production in *Datura metel* (Zahra et al., 2015) and Tanshione production in *Salvia castanea* hairy root cultures (Li et al., 2015).

Table1. Different elicitors (Biotic and Abiotic) employed enhancing metabolites production in plants

Plant species	Product	Elicitor	References
Ambrosia artemisiifolia	Thiarubrine A	*Protomyces gravidus*	Bhagwath and Hjortsø, 2000
Catharanthus roseus	Ajmalicine	Cadmium	Zheng and Wu, 2004
Arachis hypogaea	Resveratrol and trans-resveratrol	Sodium acetate	Medina-Bolivar et al., 2007
Azadirachta indica	Azadirachtin	*Claviceps purpurea*	Satdive et al., 2007
Plumbago indica	Plumbagin	Jasmonic acid	Gangopadhyay et al., 2011
Echinacea purpurea	Caffeic acid derivates	Gibberellic acid	Abbasi et al., 2012
Azadirachta indica	Azadirachtin	Fungal culture filtrates	Srivastava and Srivastava, 2014
Salvia castanea	Tanshione	Silver (Ag)	Li et al., 2015
Salvia miltiorrhiza	Tanshione	Methyl jasmonate	Xiaolong et al., 2015
Datura metel	Atropine	Bacillus cereus	Zahra et al., 2015
Datura metel	Atropine	Silver (Ag)	Zahra et al., 2015
Oldenlandia umbellate	Anthraquinones	Yeast extract	Krishnan and Siril, 2018

Hormonal elicitors i.e., methyl jasmonate and salicylic acid (plant immune signaling molecules), pectin and cellulose (constituents of plant cell wall) glucan and chitin (from microorganisms) (Namdeo, 2007; Shilpa et al., 2010; Sharma et al., 2011) are also used to enhance production. Methyl jasmonate enhanced tanshione production in *Salvia miltiorrhiza* hairy root cultures (Xiaolong et al., 2015). Jasmonic acid was used as an elicitor in hairy root cultures of *Plumbago indica* to increase production of plumbagin and results obtained with this addition were fruitful (Gangopadhyay et al., 2011). Hairy root cultures of *Withania somnifera* had shown better productivity of Withanolide A, Withanone and Withaferin A metabolites with use of Salicylic acid (Sivanandhan et al., 2013). Srivastava and Srivastava (2014) deduced that use of different fungal culture filtrates promoted azadirachtin production as the fungal derived oligosaccharides are potent elicitors in *Azadirachta indica* hairy roots cultures. Polysaccharide fraction of the yeast extract (Yeast elicitor) was used for enhancing anthraquinone production in *Oldenlandia umbellate* cultures (Krishnan and Siril, 2018). The selection of the adequate elicitor depends on the type of plant culture employed and the metabolite being produced. Zahra et al. (2015) used *Bacillus cereus* as a biotic elicitor to enhance atropine production in *Datura metel* and obtained good results. *Ambriosa artemisiifolia* hairy root cultures for Thiarubrine A production was elicited with cell wall filtrates of the plant pathogenic fungus *Protomyces gravidus* as a result enhanced production was seen (Bhagwath and Hjortsø 2000). Biotic elicitors from Claviceps purpurea were used to increase azadirachtin production *Azadirachta indica* (Satdive et al., 2007).

2.4. Cultural conditions

Inoculum density, hydrogen ion concentration, temperature, intensity and quality of light are some of the culture conditions that can affect the productivity in shake flask or bioreactors as reported in various studies.
Inoculum density is determined by investigating various optimization steps including nutrient availability, O_2 and volume of culture flask (Jeong et al., 2009). Hairy root culture of *Pueraria candollei* var. *candollei* in liquid B5 medium (50 ml) has shown that the recommended 1% inoculum size and 32^{o}C culture temperature favoured optimum growth and isoflavanoid production (Danphitasanuparn et al., 2012).
Hydrogen ion concentration: in *Silybium marianum* active biomass seen to be accumulated at pH 5 and temperature 25^{o}C , silymarin production increased as well, in comparison to the pH 6,7 (Rahimi and Hasanloo, 2016). In another study, culture medium with pH 5.8 favoured alkaloid accumulation in hairy root (Gai et al., 2015). Till date few studies have reported the effect of temperature on culture medium. However temperature plays significant role in influencing active biomass formation and secondary metabolite accumulation. The overall TAG content increases in temperature range from 24^{o} to 28^{o}C but decreases when temperature rises above 28^{o}C in cultures of *A. membranaceus* (Jiao et al., 2015). The quality, intensity as well as time period of exposure of light can affect growth of culture. For an instance, Mukherjee et al. (2016) presented that the hairy roots of *Daucus carota* under continuous illumination turned green suggesting accumulation of chlorophyll as compared to the roots grown in dark in which chlorophyll content is absent.

3. Scale-up by Means of bioreactors

Up-scaling is accomplished by means of bio reactor. Hairy roots are promising systems for secondary metabolite production. They have shown faster growth as compared to the plant cell cultures without any phytohormone in medium (Rao and Ravishankar, 2002). They have biosynthetic capacity to produce secondary metabolites at higher concentrations as compared to the parent plant as well are genetically stable (Kim *et al.*, 2002) and can be useful to study plant metabolic pathways and physiology (Hu and Du, 2006). These properties suggest that they can be used for large scale production, but being highly sensitive and delicate in nature they do possess certain challenges in creating a bioreactor with most suitable configuration. Lack of technology can be considered as a major drawback for large scale production from these roots. Bioreactors are manufactured as contained culture systems which are designed to create favourable homogenous environment i.e. aeration, ph, dissolved gas, temperature along with in/outlet for creating continuous channeling of air and liquid for the mass propagation of cells, tissues, somatic embryos or organic propagules (Stiles et al., 2013).

Characteristic features of an ideal bioreactor:

- An ideal bioreactor should maintain aseptic environment.
- It should minimize the development of shear forces while performing.

- It should create favourable homogeneous environment for the culture in order to produce bioactive compounds at a continuous and steady rate.
- There should be an efficient system for light and CO_2 availability for photosynthesis in case of mixotropic or phototropic cultures.
- It should not restrict the availability of the nutrients and oxygen to the biomass (no mass transfer limitation).
- Support matrix for the roots should be considered.

3.1. Challenges in designing a suitable bioreactor

Delicate morphology and non-uniform growth of hairy roots makes it difficult to increase the production to industrial scale (Patra and Srivastava, 2016). While growing, hairy roots generally form interlocked networks that resist the transfer of oxygen and nutrients to inner parts resulting in non-homogeneous environment that give rise to necrotic patches (Vashishtha and Sharma, 2015). They are highly sensitive in nature as they respond to even slightest changes in environment like temperature or oxygen (Halder et al., 2018). These changes lead to morphological changes in the roots i.e. thickness, root length and density affecting the productivity of the secondary metabolites (Srivastava and Srivastava, 2012). Vigorous mixing in these reactors by means of the impellers though increases the overall availability of the nutrients as well as oxygen, but the increase in hydrodynamic shear stresses can cause reduction of the vitality of the hairy roots since they are highly delicate in nature leading to the formation of callus which causes hindrance and eventually reduction of the secondary metabolite production hence isolation of the growing roots from the impeller can be useful (Patra and Srivastava, 2012). In some cases perfect sterile conditions for longer periods is the primary requirement for the growth (Patra et al., 2016). In case of light dependent formation of secondary metabolites, exposure of light to culture can affect the activity of the enzymes responsible for pigment production (Shin et al., 2002). Not all cultures require light. Support matrix for roots can enhance biomass accumulation (Srivastava and Srivastava, 2012). It is also proposed to initially grow roots in liquid phase system until the root tips are distributed uniformly throughout the packaging matrix and then run the reactor in the gas phase (Ramakrishan et al., 1994). With increasing volume the pressure on hairy roots increases because of their own weight. Hence size of the container and volume of the medium in bioreactor for growth are also important factors to consider (Neelwarne and Thimmaraju, 2009).

4. Types of bioreactors

Bioreactors are generally classified into three main categories; liquid phase reactors, gas phase reactors, or hybrid reactors (a combination of both liquid and gas phase) (Kim et al., 2002). For hairy root cultures, bioreactors can also be classified as agitated and bed reactors.

4.1. Liquid phase reactors

In these reactors hairy root biomass in completely submerged in the liquid media. Mass transfer of gaseous media is a rate limiting factor in these reactors. Stirred tank reactors, bubble column reactors, airlift reactor, submerged convective flow reactors etc. are some of the examples of liquid phase reactors.

4.1.1. Stirred tank reactors

It is a liquid phase with a mechanical agitator. Motor operated impeller is specifically designed to fulfill the demand of oxygen in aerobic cultures (Mishra and Ranjan, 2008), on impeller region an aeration device is placed to provide well dispersed gas phase in liquid phase. Though being heavily used in biotechnology, as for hairy root culture these reactors are generally not suitable as hairy roots are highly delicate. High shear stresses caused by impeller rotation can result in wounds and callus formation ultimately affecting overall productivity. For example , in hairy root cultures of *Azadirachta indica* for Azadirachtin production, different liquid phase bioreactors were used to compare the productivity. After 25 days, no hairy root formation as well as high phenolic content indicated that this is because of the damage caused by aeration and agitation by impellers of the conventional stirred tank reactor (Srivastava and Srivastava, 2013). Hence it was suggested to reduce the power and speed of impeller rotation.

Hilton and Rhodes (1990) modified a 14 L conventional stirred tank reactor using stainless steel cage which helped in separating the roots from the stirrer. In continuous mode at 30^{o}C, production rate of upto 8.3 mg hyoscyamine per liter per day was achieved. This suggested that separation strategy can give fruitful results. In another experiment, production of metabolite artemesinin was successfully increased in cultures of *Artemisia annua* by using steric impeller in a 3L reactor. After 25 days of cultivation under optimized conditions, total accumulation of 6.3 g/L dry weight (37.50 gm fresh weight) of biomass and 0.32 mg/g of artemisinin content was seen (Patra and Kumar, 2014). In cultivation of highly dense hairy roots of *Azadirachta indica* a 3L stirred tank reactor was modified using steric impeller and polyurethane foam (PUF) disc for supporting hairy roots because they are self- immobilizing. After 25 days, there was 15.2 g/l of biomass and 6.4 mg/g (97.28 mg/L) of azadirachtin production (Srivastava and Srivastava, 2012).

4.1.2. Bubble column reactor

This reactor belongs to general class of multiphase reactors. In these reactors hairy roots are dispersed in liquid medium in a vertical column. For mixing/agitation purpose, a bottom located air distributor or sparger is present that generates an up flow of air bubbles. Since no mechanical agitation is involved, effect of shear stress decreases. In hairy roots of *Beta vulgaris*, the pigment production and biomass accumulation were tested in a 2L bubble column reactor for scale up. Results indicated mass transfer limitation which lowered yields than the experimentation conducted in shake flasks (Mukundan et al., 1998). In hairy root cultures of *Cinchorium intybus L.* for scale up, the reactor was modified for the immobilization of the roots using a vertical plastic basket which resulted as an efficient method in order maintain even distribution of roots throughout the vessel and enabled multidirectional root growth pattern (Bais et al., 2002). In hairy root culture of *Artemisia annua*, resulted biomass concentration of 15.3 DW/L was obtained in a bubble column reactor which was better as compared to the biomass concentration in a mist reactor (Kim et al., 2002). Influence of elicitors, methyl jasmonate and polyamine was investigated for the production of metabolite betalain in *Beta vulgaris* hairy root cultures in a 3L bubble column reactor modified with an anchorage and a plastic (Suresh *et al.,* 2004).

Production of betacyanins and betaxanthins in red beet hairy root cultures was monitored in this reactor designed with two step basket. The supply of air bubbles kept in continuity from the bottom of the reactor. Resultant biomass production was 1.2 times higher as compared to one step basket (Neelwarne and Thimmaraju, 2009). Srivastava and Srivastava (2013) modified a bubble column reactor by incorporating a polypropylene basket and in another case placed polyurethane foam disc as root support. Results proved that modified reactor favoured higher metabolite and biomass production and reactor with polyurethane foam

proved to be a suitable configuration. In another attempt, a polypropylene mesh support was used in a bubble column reactor and a polyurethane foam support was used in another in hairy root cultures of *Catharanthus roseus* for the production of metabolite ajmalicine to show which supported better productivity. Polyurethane foam support bioreactor resulted in accumulating 34±2.3 mg/L of ajmalicine for 1.13 mg/L of total productivity (Thakore et al., 2017). Utilization of cell bags (plastic disposable chambers) also became popular as they minimize the time consuming cleaning as well as labour costs. They can be considered as the future low cost bioreactors for hairy root cultures (Eibl and Eibl, 2006).

4.1.3. Airlift reactor

Airlift reactors are generally described as bubble column reactors with a draft tube. In this reactor the supply of air is from bottom of a central draft tube which controls air circulation in medium. Use of this tube separates the upward and downward flow. Conventional airlift reactors have been used in scale up cultures of hairy roots. But they are not capable of producing optimum yield since most common problem with liquid phase reactors is oxygen mass transfer which can limit the proper growth of hairy root lines. Vigorous agitation and shear stress can cause structural disorganization and ultimately callus formation. Hence biomass productivity decreases to non profitable level. More importantly the excessive gas-phase channelling and the uneven root distribution at certain region in air lift reactors will cause the hindrance in liquid flow because of clump formation (Kim and Yoo, 1993), but if roots can be immobilized than HR biomass increases eventually (Kondo et al., 1989)
They possess lower shear stresses as compared to stirred tank reactor. The density difference between riser and down comer facilitates liquid circulation with high turbulence. Production of Puerarin in *Puraria phaseoloides* hairy root cultures in 2.5 L air-lift bioreactor increased 200 times resulting 5570 µg/g dry wt. puerarin as compared to 250 ml shake flask in 3 weeks (Kintzois et al., 2004). Use of draft tube can prevent the coalescence of bubbles as they move in one direction distributing the shear stresses equally throughout the reactor providing much stable environment. In *Artemisia annua* hairy root culture, production of metabolite artemisinin was carried out in a modified air lift reactor (inner loop) with stainless steel meshes placed throughout the column which yields 577.5 mg/L after 20 days interval of time (Liu et al., 1998)

4.2. Gas phase reactors

In these reactors gas is present in continuous phase and liquid is the dispersed phase. Cultured hairy roots are directly exposed to air and nutrients in the form of liquid, are either sprayed onto the roots or delivered in the form of mist. These reactors are capable of eliminating any oxygen deficiency in dense root beds as well as reduce shear stresses to manageable rates. Liquid dispersed bioreactor, radial flow bioreactor, droplet phase bioreactor are some of the gas phase bioreactors.

4.2.1. Liquid- dispersed/ Bed reactors

Liquid dispersed or bed reactors or trickle bed reactor, here the strategy of dispersing the mixture of air and nutrient medium on the bed of the roots with the help of spray or in droplet or mist mode. Media that remains unused is collected and re-circulated in similar way. In comparison to the liquid phase reactors, they are of greater use as they reduce the problem of oxygen mass transfer to considerable extent as well as provide low shear stress environment suitable enough to cultivate hairy roots. Nutrient sprinkle, nutrient mist, nutrient droplet or trickle-bed or spray reactor (Huang et al., 2004; Kuzma et al., 2009; Jaremicz et al., 2014) are types of liquid dispersed reactors categorized on the basis of the dispersed size of the droplets that vary from 0.01 – 10 µm and in other reactors size can increase from 10 µm (Weathers *et al.*, 2008). Apart from a reactor, a mist reactor system consists of a heating system, the mist generator system and a control system to check oxygen flow. For heating purpose a polymer thick film (PTF) heater is present that is capable of rapid temperature switching. The mist generator system creates mist spray, for proper flow of oxygen; an oxygen flow control system is present. All of the components are connected to the reactor and mist inducer (Cham et al., 2016). Both bubble column and mist reactors were used in *Artemisia annua* hairy root cultures for comparative study. Results showed that threefold biomass was produced in mist reactor as compared to bubble column (Kim et al., 2001). Mist provides larger surface area for oxygen mass transfer plus these reactors can facilitate fast replenishment of the nutrients and removal of toxic products as well. Absence of agitator leads to lower shear stresses making them more prominent as appropriate reactors for this approach.
Azadirachtin production from hairy root cultures of *Azadirachta indica*, gas phase reactor (nutrient spray and mist reactor) was used in order to increase the overall production. Batch wise cultivation carried out for 25 days yielded 27.4 mg/l of Azadirachtin from 9.8g/L dry wt. accumulated biomass (Srivastava and Srivastava, 2012). Artificial Neural Network (ANN), a modern biological neural network system based approach is used to work out the culture parameters and operating conditions i.e. size of the inoculum, media volume ,initial packing density, initial concentration of sucrose in the medium and mist ON/OFF time, time of culture (Osama, 2013). ON/OFF system is helpful in controlling the transport of nutrients to the cultures transformed roots in reactor (Osama et al., 2013, Ranjan et al., 2009).

4.3. Hybrid reactors

Gas phase reactors are advantageous as compared to the liquid phase reactor but gas phase reactors have some limitations too. Problem being, there is no possible way of distribution of the roots without manual loading. Hybrid reactors overcome this limitation. Hence proposed solution was the combination of both the reactors. In a case study, initially bubble column reactor was used to suspend the roots; packing rings were used as support allowing uniform distribution. In about two weeks the root clumps became dense. Then reactor is switched to trickle bed. This led to the exposure of roots to gaseous environment. In this case the roots were chopped in blender; formed slurry is pumped into the reactor. Hence the manual labor destined for cutting and inoculation purposes is eliminated. An ideal bioreactor should exhibit the properties i.e. minimal shear stress, homogenous culture environment, high oxygen mass transfer activity for highest biomass production possible and better growth characteristics in roots. Future of hairy roots commercialization highly depends on low capital as well as operating cost. This area is open for developments which can be made in near future.

CONCLUSION

Hairy root culture can be obtained from all plant species of economical and pharmaceutical importance. Therefore, in order to conserve the plant biodiversity and for producing bioactive compounds from them, this technique could conclusively be explored for rare and endangered medicinal vegetation. In addition, HRCs have become effective technology for understanding the biosynthesis pathway of plant-derived important compounds and considered to be suitable production system having various advantages over cell suspensions and field grown plants. Hairy root cultures are also known to be practical model system for the production of specialized metabolites or recombinant proteins and

unraveling the biological processes involved in the plant for enhancing the metabolites production. The Bioreactors are an ideal resource to increase overall production of metabolites which provides the controlled systems for the growth and reproduction of hairy roots and monitor them throughout the process. But mass production is inherently challenging because the process is complex and requires thorough knowledge. The study of several optimization steps has led to advances in the design and construction of bioreactors, leading to the production of several secondary metabolites in several plant species. In order to produce the required secondary metabolites, many factors must be considered, because hairy is fundamentally very fragile, so their amplification through a bioreactor can be challenging.

ACKNOWLEDGMENTS

The authors thank Head, Department of Plant Sciences, Central University of Himachal Pradesh for providing necessary facilities. S is thankful to Central University of Himachal Pradesh for financial support in the form of fellowship.

AUTHOR CONTRIBUTIONS

MS suggested the idea and designed the study. S collected the data and involved in draft preparation for the review. The final editing was done by MS and approved the final version.

COMPETING INTERESTS

The authors declare they have no conflict of interest. The manuscript has not been submitted for publication in other journal.

ETHICS APPROVAL

Not applicable.

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