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Mass production of Ajmalicine by bioreactor cultivation of hairy roots of *Catharanthus roseus*

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ABSTRACT

Catharanthus roseus has been known to produce Ajmalicine which is used for treatment of circulatory disorders. Significantly low content of the drug in the natural plant necessitates development of alternative production protocols. Hairy root propagation was considered as a viable alternative. Mass cultivation of the hairy root culture was attempted in several bioreactor configurations; Bubble column, Rotating drum bioreactor, Modified Bubble column with polypropylene (PP) mesh support and Modified Bubble column with Polyurethane foam (PUF) support. Important factors like medium composition, inoculum density, illumination period and aeration rates were optimized before mass propagation of hairy roots. From the data gleaned, the cultivation in rotating drum bioreactor resulted in ajmalicine content of only 4.6 ± 0.4 mg/l. However, the ajmalicine concentration surpassed even that obtained in shake flask (with 34 ± 2.3 mg/l) in a customized bioreactor wherein the hairy roots were anchored onto a polyurethane foam, the highest reported in this scale of cultivation.

1. Introduction

Catharanthus roseus is a medicinal plant belonging to the family *Apocynaceae*, which is one of main sources of Ajmalicine, with extensive medicinal application in the treatment of circulatory disorders and enhanced blood pressure [1]. Hypertension has been identified as a major public health issue as it accounts for 9.4 million deaths across the globe more so because it rarely shows any symptoms in the early stages and goes undiagnosed in most of the patients. Of the total deaths due to heart diseases 45% are attributed to hypertension. About 45% of adults worldwide in the age group of 25 and above are a victim of this condition [2].

Plant derived extracts have been extensively used as a component of healthcare, cosmetics as well as dietary supplements [3,4]. Currently around 10,000 medicinal plants have been identified as threatened species [5]. The chiral and complex structure of the secondary metabolites makes the chemical synthesis difficult [6]. Engineered cell and/or organ cultures cultivated *in-vitro* are preferred since the manipulations are faster and reliable in these cases owing to fewer environmental influences [7]. As opposed to plant cell suspensions hairy roots, derived from the infection of gram negative soil bacterium *Agrobacterium rhizogenes*, can serve as an alternative means for the production of commercially important biomolecules. Due to the low unstable content in the native plant and loss of viability with periodic subcultures in suspension cultures, biosynthesis from hairy roots is considered desirable [5,8]. Moreover several compounds are accumulated only in differentiated cultures at different developmental stages [9].

Once optimized in shake flask, hairy roots can be scaled up to industrial level forming an abundant sustainable source of bioactive molecules. The likelihood of successful bioreactor design and operation is governed by the biological and engineering demands of the system which includes a number of factors: oxygen transfer and mixing, low shear and hydrodynamic forces and an effective control of the physico-chemical environment [6,10]. Reportedly hairy roots are oxygen limited even in shake flasks [11]. Nevertheless, significant work has been done in development of liquid phase bioreactor systems for a wide range of species [12,13]. Mass propagation has also been attempted in liquid dispersed bioreactors like mist and trickle bed for some hairy root cultures but have met with mixed responses [14,15].

The commercialization of this technology is still in its infancy with so far two private companies ROOTec, Basel Switzerland (http://www.rootec.com) (a Swiss private company started in 2008) and Root lines Technology SAS (http://www.rootlines-tech.com), a French firm, having moved from bench scale to an industrial

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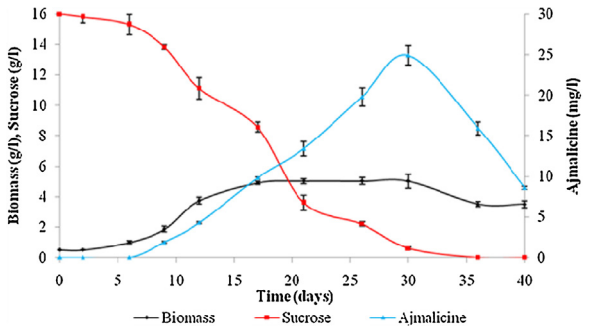

Fig. 1. Study of growth and production kinetics in shake flask hairy root culture.

level production. More recently a bench-top bioreactor allowing continuous extraction of secondary metabolites was designed for the cultivation of *C. roseus* cell suspensions [16]. According to databases (SCOPUS) as on July 2014 there are about 2748 reports on hairy root cultures. These include 2446 research articles and 109 reviews (for keyword: hairy root) covering various areas (source: http://www.scopus.com) of which 148 reports (including 132 articles and 9 reviews) are for *Catharanthus roseus* hairy roots.

Despite several laboratory scale success stories involving mathematical model based fed batch and inclusion of several yield enhancement strategies [17,18] till date only three reports of mass cultivation of *C. roseus* hairy root culture are available of which two analyse ajmalicine production [19,20]. In view of the above factors the present investigation focused on the use of different bioreactor configurations for mass scale synthesis of Ajmalicine from hairy roots.

2. Materials and methods

2.1. Seed inoculum preparation and maintenance

Hairy root cultures of *Catharanthus roseus* (L.) G. Don cv. Prabal, clone CP 32 [21] to be used as inoculum in shake flask and concurrent bioreactor studies were subcultured every 15 days in 250 ml Erlenmeyer flasks containing 40 ml of optimized Gamborg's B5/2 medium, pH 5.8 on a gyratory shaker rotating at 70 rpm with 16/8 h L/D photoperiod at 25 ± 1°C.

For conditioning of the medium initial screening of the various components was done by Plackett Burman [22]. Optimized concentrations were thereafter achieved by Response surface methodology facilitated by Design Expert (version 5.0.9) software (Stat-Ease Corporation, USA). In brief the statistically optimized medium recipe was as follows: 2.75 g/l of Potassium nitrate, 37.5 mg/l of Sodium hydrogen monophosphate along with Gamborg B5/2 Major salts + B5/2 Minor salts + B5 vitamins + 16 g/l sucrose. For generating ajmalicine and biomass production statistics and sucrose consumption profile, the hairy roots in shake flask were harvested every 3 days from different flasks in triplicates for a period of 40 days (Fig. 1).

2.2. Analysis of optimal inoculum density

In order to determine the inoculum density that would promote both root growth and secondary metabolite synthesis different concentrations based on the dry weight (DW) (0.5, 1.25, 2.5, 3 g/l) were tested. The Ajmalicine and biomass values were assessed in triplicates. Table 1 indicates the average values of three experiments.

Table 1
Effect of inoculum size on biomass and Ajmalicine accumulation *(Average of triplicate experiments).*

Inoculum size (DW)	Ajmalicine (mg/g)	Biomass (g/40 ml)FW
0.5	0.44	2.2
1.25	0.21	1.6
2.5	0.26	1.1
3	0	0.93

FW/DW ratio: 10.6.

Table 2
Effect of photoperiod on biomass and Ajmalicine accumulation (*Average of triplicate experiments*).

Illumination conditions	Ajmalicine (mg/g)	Biomass (g/l)
16/8 h L/D	0.39	1.9
Dark	0.16	1.4

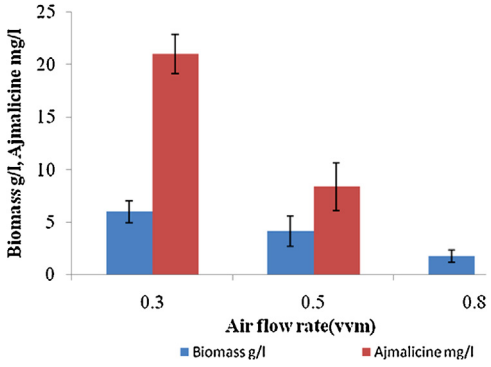

Fig. 2. Effect of air flow rate on biomass and Ajmalicine accumulation in hairy root culture of *C.roseus.*

2.3. Analysis of photoperiod

Hairy roots of *C. roseus* were incubated under two different illumination conditions: 16/8 h light/dark (L/D) and complete (24 h) dark (D) conditions. The experiments were conducted in triplicates in 40 ml of half strength Gamborg B5/2 medium +0.3% w/v phytagel +3% (w/v) sucrose and pH 5.8. Both light and dark adapted cultures were harvested after 30 days. Average values of biomass and ajmalicine are as indicated in Table 2.

2.4. Analysis of aeration rate

Sterile filtered air was bubbled at different rates (0.3, 0.5, 0.8 vvm) into a customized reagent (1L) bottle incubated on a gyratory shaker rotating at 70 rpm using optimized medium (200 ml) of the composition and conditions mentioned previously. The vvm was based on the total volume of the medium (Fig. 2).

2.5. Bioreactor operation

Batch cultivation of *C. roseus* hairy roots was initiated in a 3 l bubble column bioreactor (Applikon Dependable Instruments, The Netherlands) (Fig. 3) equipped with a mesh sparger. Air sparging facilitated both aeration and mixing. The roots remained submerged in the medium throughout the cultivation. Such an arrangement would feature less shear on the growing roots [6,23].

Taking into consideration the problems in the conventional bubble column bioreactor design several modifications were opted. Different supports/anchorage suiting the special requirement of the root species promoting growth and synthesis were custom fabricated. The rotating drum configuration consisted of a 7 l stainless