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SHORT COMMUNICATION

Suitability of bench scale bioreactor system for shoot biomass production and bacoside biosynthesis from *Bacopa monnieri* (L.)

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According to folklore, *Bacopa monnieri* commonly called as Brahmi is known for its cognitive enhancing properties. The plant is found abundantly in wetlands but the drug content (bacosides) is very low (0.2%), therefore, alternative biotechnological protocols are highly needed to supplement the constant source of this valuable plant material which produces stable amounts of bacosides. The present study was conducted to explore the application of different culture systems for cultivation of shoot biomass and maximization of biologically active bacoside biosynthesis in this medicinally important plant. Shoot cultures of Bacopa were cultivated in two different modified benchtop bioreactors: glass bottle bioreactor and balloon type bubble bioreactor and compared with those grown in traditional Erlenmeyer agitated flask. The shoots cultivated in the balloon type bubble bioreactor system showed excellent growth (growth index 796.47 ± 17.27 fresh weight and 395.55 ± 7.55 dry weight) as compared to glass bottle bioreactor system (growth index 488.17 ± 14.4 fresh weight and 327.79 ± 6.64 dry weight) and agitated flask (growth index 363.43 ± 11 fresh weight and 304.22 ± 6.76 dry weight). Furthermore, bacosides produced by shoot cultures cultivated in the balloon type bubble bioreactor (321.95 ± 17.14 mg/L) and glass bottle bioreactor (180.18 ± 6.25 mg/L) configurations were ∼2.78 fold and ∼1.55 fold higher than that recorded in agitated flask cultures (115.7 ± 3.84 mg/L). The balloon type bubble bioreactor system was found to be advantageous for enhancing *B. monnieri* shoot biomass and bacoside biosynthesis along with ensuring a successful protocol for continuous supply.

KEYWORDS
bacoside biosynthesis, biomass, bioreactor cultivation, brahmi, nutrient consumption

1 | INTRODUCTION

Bacopa monnieri (L.) Wettst. commonly known as Brahmi, Neer-brahmi, Jal brahmi, water-hyssop etc. possess a wide array of therapeutic activities including nootropic action in humans due to presence of bioactive compounds including triterpenoid saponins called bacosides (A & B) [1,2]. Bacoside A is the main bioactive saponin responsible for the memory-enhancing effect of *B. monnieri* [3]. Due to the memory enhancing property of bacosides, the demand for this herb has increased world-wide for its use in several commercial preparations which led to its depletion from the natural habitat [4], making brahmi an endangered species [3]. In order to meet the growing market demand for the plant biomass for bacosides biosynthesis, it becomes important for the development of alternative production methods using tissue culture strategies [5].

Abbreviations: AF, agitated flask culture; BTBB, balloon type bubble bioreactor; DW, dry weight; FW, fresh weight; GBB, glass bottle bioreactor; GI, growth index; MS, Murashige and Skoog.

The utilization of bioreactor is an exciting preposition in medicinal plant biotechnology for shoot multiplication, and production of bioactive metabolites has recently progressed exhibiting a considerable development towards cultivation of plants for metabolite production using cost effective, easily available bioreactor devices [6–10]. The potential of in vitro regenerated shoot cultures for the production of bacosides [1, 11–13] along with suitability of liquid medium to grow shoots has been analyzed [14,15], which provides the basis for large scale cultivation in bioreactor. Bacoside biosynthesis during in vitro shoot multiplication of *B. monnieri* grown in Growtek and air lift bioreactor has been reported earlier by our research group [16]. Since bioreactor plays an important role for scaling of metabolites production under in vitro conditions, the present study evaluates suitability of modified bench scale bioreactor for production of shoot biomass and bacosides in *B. monnieri*. The exploitation of production systems in vitro could be considered as a viable alternative to reduce the pressure on existing natural population of *B. monnieri* and make available a constant supply of bacosides for use in the pharmaceutical industries.

PRACTICAL APPLICATION

The current study is first of its type which provides a sustainable and an efficient benchmark for large scale shoot production of highly acclaimed medicinal plant *Bacopa monnieri* as a reliable and continuous source of memory enhancing bacoside metabolites. This study was conducted in lieu to suffice the high commercial demands of this highly valuable bioactive metabolite in medico-pharmacology markets worldwide.

2 | MATERIALS AND METHODS

2.1 | Plant material

Multiple shoot cultures of *B. monnieri* were initiated and maintained on MS (Murashige and Skoog) [17] medium as explained earlier [16]. Proliferated shoot cultures were subcultured onto liquid medium of same composition and the shoots of about 1–1.5 cm long with healthy leaves (after one month of growth) were used as inoculum for the cultivation in different culture systems.

2.2 | Bench scale configurations for enhancing bacoside biosynthesis

Two type of in vitro culture systems, glass bottle (1 L) and separating funnel (2 L), were fabricated as aerated bioreactor (Figure 1). The bioreactors were steam sterilized at 121°C for 15–20 min.

2.2.1 | Agitated flask (AF, constant immersion with agitation)

A 1 L Erlenmeyer flask (Borosil; OD × ht 131 × 220 mm, neck OD 42 mm) was used to grow shoots in liquid medium. The culture flask was tightly closed with muslin cloth wrapped plugs to guarantee air sterility (ure 1A,B). The average ∼5.66 g excised shoots having ∼3.86 g fresh weight (FW) were aseptically inoculated into the Erlenmeyer flask containing 100 mL of liquid medium.

2.2.2 | Glass bottle bioreactor (GBB, constant immersion with aeration)

An autoclavable 1 L glass bottle (Schott Duran, USA) (height 220 mm, base diameter 80 mm) was used. The system was closed with a screw cap having four connection ports (diameter 5 mm each). Two of the ports were locked with screw caps to guarantee air sterility. One of the ports was modified for air inlet connected with silicone tubing (diameter 5 mm) and hydrophobic filters 0.2 μm PTFE (autoclavable, PALL® Corporation, USA). Bubble free air was provided through the silicon tubing at 0.5 vvm (air volume/culture medium volume/min) after being passed through a pre-filter (hydrophobic 0.2 μm PTFE). Overpressure from the vessel was relieved while maintaining asepsis by passing air from the system through an exhaust port connected to a 0.2 μm filter. The caps and all individual components made of polypropylene (PP) were completely autoclaved together with the medium contained in the bottle, so that they can be reused repeatedly (Figure 1C,D,E). The GBB containing 100 mL of liquid MS medium was inoculated with ∼8.66 g excised shoots having ∼4 g FW aseptically through the inoculation port (32 mm diameter) and maintained under uniform culture conditions.

2.2.3 | Balloon type bubble bioreactor (BTBB, constant immersion with aeration)

A globe shaped 2 L separating funnel with plain stem (Perfit, India) (Corning glass, height 380 mm, stem length 95 mm) with a wide mouth was modified into a bioreactor system. The system was closed with a silicon cork (bottom 28 mm × top 36 mm × height 30 mm). Two holes were perforated in the silicon cork to serve as inlet/outlet for air through silicone tubes (5 mm) fitted with hydrophobic filters (0.2 μm). The cultures were aerated at 0.5 vvm through an L- shaped glass sparger (height 300 mm) using an oil free air compressor connected to a Rotameter (air flow control system) to regulate air volume and fitted with a pre-filter (hydrophobic 0.2 μm PTFE) to ensure air sterility (Fig. 1F,G,H). The BTBB

FIGURE 1 Shoot biomass of *B. monnieri* in various culture systems: (A, B) 1 L agitated flask; (C–E) 1 L glass bottle bioreactor assembly with shoots; (F–H) 2 L balloon type bubble bioreactor assembly with shoots

containing 200 mL of liquid MS medium was inoculated with ~12.66 excised shoots having ~5.91 g FW aseptically and maintained under uniform culture conditions.

2.3 | Culture conditions

Both the bioreactors were incubated in the culture room at 24 ± 2°C, illuminated by cool white fluorescent tube-lights (3000 lux) with a 16 h photoperiod for a culture cycle of 4 weeks. The agitated flask cultures were placed on a rotary shaker (New Brunswick, USA) at 100 rpm and incubated in optimum growth chamber. The shoots from the agitated flask culture were used as the control.

2.4 | Biomass determination and analytical procedures

The shoot cultures were harvested after 4 weeks and the growth parameters such as shoot multiplication rate, FW, DW, and growth index (GI) were determined. The shoot biomass obtained from all systems was blotted dry on Whatman filter paper and weighed (FW). The biomass was then air dried to constant weight to determine DW. Growth in terms of GI was calculated: GI = (Final weight–Initial weight) / Initial weight × 100. For bacosides analysis, shoot biomass (dried shoot) was extracted using HPLC grade methanol. Quantitative analysis was carried out by HPLC using Agilent-1100 series HPLC system. Change in conductivity, pH, and nutrient depletion in the spent liquid medium (i.e., medium left after culture cycle of 4 weeks) in all systems were also measured. The details of the procedures were as reported earlier [16]. The bacoside biosynthesis was expressed as milligram per litre (mg/L) of dry weight.

2.5 | Data analysis

The results are expressed as mean values of three independent experiments. Data was analyzed by one-way analysis of variance (ANOVA) using statistical software SPSS version 20 (SPSS Inc., Chicago, IL, USA). The significance of differences among means was analyzed using Duncan's multiple test at $p \leq 0.05$.

3 | RESULTS AND DISCUSSION

Bacopa monnieri shoots inoculated in both the bioreactors started to multiply vigorously after one week interval and varying increase in biomass was recorded, as shown in Figure 1. The type of culture systems used influenced the growth behavior of shoots.

3.1 | Biomass accumulation and bacoside biosynthesis

The biomass growth and bacoside biosynthesis in bioreactor were determined after 4 weeks cultivation, in parallel to the agitated flask culture (Table 1). The highest accumulation of biomass was achieved in both bioreactors BTBB (264.33 g/L FW and 30.41 g/L DW) and GBB (235 g/L FW and 22.35 g/L DW) than that in AF (179.2 g/L FW and 20.6 g/L DW), probably due to better hydrodynamic environment in the bioreactor, which led to better availability of the main nutrients of the medium at the optimal conditions of cultivation (Table 1). The BTBB and GBB grown shoots were healthy, fully green, and had well-developed expanded leaves with rooting at the base. The shoots cultivated in the BTBB showed excellent growth (GI 796.47 FW and 395.55 DW) followed by growth recorded in GBB (GI 488.17 FW and 327.79 DW) and agitated flask (GI 363.43 FW and 304.22 DW). The results are in accordance to earlier similar findings where organ culture of *Lavandula officinalis* was cultivated in 5 L bubble column bioreactor to produce rosmarinic acid [18]. Similar reports on cultivation of shoot cultures of *Spathiphyllum cannifolium* [19] and *Stevia rebaudiana* [20] in bubble column bioreactor in order to produce flowers and biomass, respectively have been reported. Reports are available wherein bioreactor cultivation for shoot cultures of *Artmissia annua* [21] and other medicinal plants has been demonstrated [22]. Difference in shoot multiplication rate was also observed between cultures in the BTBB (5.01), GBB (4.32), and AF (2.6). The results obtained in the present study are comparable with our earlier reports [16] where airlift bioreactor showed better growth in comparison to shake flask culture.

HPLC analysis revealed higher bacoside content and biosynthesis in BTBB (9.34 mg/g DW and 321.95 mg/L DW) followed by GBB (8.15 mg/g DW and 180.18 mg/L DW) in comparison to agitated flask (5.62 mg/g DW and 115.7 mg/L DW) (Table 1). The higher content of bacosides for both BTBB and GBB might be attributed to the greater availability of oxygen due to the application of external aeration and lesser physical stress as compared to long-term submerged liquid cultivation in agitated flask. Similar to present observations, suitability of various types of bioreactor for shoot culture cultivation and metabolites accumulation for medicinally important plants has been reported, polyphenols by *B.monnieri* shoots [23], isoquinoline alkaloids (galanthamine) [24], phenolics and flavonoids [25], and dibenzocyclooctadiene lignans [26].

3.2 | Conductivity and pH measurement

A decrease in conductivity in the BTBB spent medium was observed (3.03 mS/cm) followed by GBB (3.39 mS/cm) in comparison to control agitated flask (1.82 mS/cm) culture

TABLE 1 Growth and bacoside biosynthesis in shoots of *B. monnieri* cultivated in different bioreactor system

Parameters		Agitated flask (1 L)	Glass bottle bioreactor (1 L)	Balloon type bubble bioreactor (2 L)
Medium volume		100 mL	100 mL	200 mL
Shoot multiplication rate		2.6a ± 0.14	4.32b ± 0.29	5.01c ± 0.09
Biomass (g/L)	FW	179.2a ± 4.87	235b ± 4.04	264.33b ± 14.53
	DW	20.6a ± 0.42	22.35a ± 1.22	30.41b ± 3.27
Growth index	FW	363.43a ± 11	488.17b ± 14.4	796.47c ± 17.27
	DW	304.22a ± 6.76	327.79a ± 6.64	395.55b ± 7.55
Bacoside A_3	mg/g DW	3.37a ± 0.03	4.73b ± 0.44	5.23b ± 0.48
	mg/L DW	69.52a ± 1.88	104.55b ± 3.48	180.39c ± 10.88
Bacoside A_2	mg/g DW	2.24a ± 0.05	3.42b ± 0.33	4.11b ± 0.38
	mg/L DW	46.2a ± 1.94	75.63b ± 2.78	141.56c ± 6.63
Total bacoside[a]	mg/g DW	5.62a ± 0.08	8.15b ± 0.76	9.34b ± 0.85
	mg/L DW	115.76a ± 3.84	180.18b ± 6.25	321.95c ± 17.14

Data represents mean ± SE of three independent experiments (three bioreactor run); mean within rows followed by different letters are significantly different at $p \leq 0.05$, (one way ANOVA, Duncan Multiple Test). Bacoside productivity (mg/L) was calculated using formula: bacoside content (mg/g DW) × dry weight of shoots per volume of culture medium (g/L)

[a]Sum of bacoside A_3 and A_2

TABLE 2 Change in pH, conductivity, total sugar, and nitrogen content of spent medium after the harvesting of *B. monnieri* shoot biomass at a culture period of 4 weeks

Parameters	Agitated flask (1 L)	Glass bottle bioreactor (1 L)	Balloon type bubble bioreactor (2 L)
pH	5.3c ± 0.012	4.59b ± 0.006	4.17a ± 0.015
Conductivity (mS)	1.82a ± 0.015	3.39c ± 0.03	3.03b ± 0.029
Total sugar (%)	0.12a ± 0.012	0.6b ± 0.034	0.57b ± 0.02
Nitrogen (%)	0.005a ± 0.001	0.01b ± 0.001	0.02c ± 0.001

Data represents mean ± SE of three independent experiments. Mean within rows followed by different letters are significantly different at $p \leq 0.05$, (one way ANOVA, Duncan Multiple Test)

medium after culture cycle of 4 weeks (Table 2), it might be possible due to selective utilization of mineral nutrients by the shoot culture for growth. A decrease in the conductivity of the nutrient medium during biomass synthesis has been observed [27]. The pH of the medium during the cultivation period declined from 5.84 to 4.17 in BTBB and 5.84 to 4.59 in GBB (Table 2). The results obtained are in line with earlier study [28] where change in pH of bioreactor (BTBB) culture medium during growth of bulblets of *Lilium* Oriental Hybrid 'Casablana'was reported. The decrease of pH in culture medium might be attributed to selective uptake of nitrate (NO_3^-) or ammonium (NH_4^+) ions as nitrogen source from the medium by plant tissue/cell and release of H^+ ions into the medium results in the acidic conditions [29].

3.3 | Nutrient consumption

A significant change in the sugar and nitrogen content in control cultures and the bioreactor systems was observed (Table 2). Measurements of nitrogen concentration in culture medium of BTBB (0.02%) and GBB (0.01%) reported slight decrease suggesting that the cultures consume much nitrogen supplied in comparison to control AF (0.005%). Lian et al. [29] analysed the dynamics of various nutrient compounds during *Lilium* bulblet growth in BTBB and observed the depletion of nitrogen (ammonium, nitrate) and sugar in the medium. Presence of lower sugar content in medium of BTBB (0.57%) and GBB (0.60%) could be attributed to continuous aeration of bioreactor which increased the photosynthetic capacity of shoot cultures (Table 2). Our results are in line with earlier investigations where nutrients in bioreactor culture medium were not fully depleted [30]. The results of our previous study [16] also revealed a decrease in nitrogen and sugar consumption by the aerated shoot biomass, probably due to the increased photosynthetic activity by shoot growth. Decrease in the sugar and nitrogen content in modified glass-column bioreactor medium used for cultivation of *Leucojum aestivum* shoot culture and galanthamine production has also been reported [24].

4 | CONCLUDING REMARKS

In conclusion, *B. monnieri* shoots grown in BTBB produced high yields of bacosides and can be used as a source of these pharmacologically active compounds. Furthermore, shoot biomass growth was significantly high in bioreactor system which also reduced the time for recovery of *Bacopa* plants. The presented results enable new perspectives in designing cost efficient bioreactor configurations that allow more effective scaling up cultivation of biomass and bacoside biosynthesis.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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