Biotechnology Journal International

21(4): 1-15, 2018; Article no.BJI.43510
ISSN: 2456-7051
(Past name: British Biotechnology Journal, Past ISSN: 2231–2927, NLM ID: 101616695)

Cost Effective Pilot-scale Ajmalicine Production by *Catharanthus roseus* Cell Suspension Cultures in a 100 Lit Bioreactor

Devanand P. Fulzele[1*] and Ajay G. Namdeo[2]

[1]Plant Biotechnology and Secondary Metabolites Section, NA&BTD, Bhabha Atomic Research Centre, Mumbai 400085, India.
[2]Department of Pharmacognosy, Poona College of Pharmacy, Bharati Vidyapeeth University, Pune 411038, India.

Authors' contributions

This work was carried out in collaboration between both authors. Author DPF has designed the bioreactor. Authors DPF and AGN designed the study and perform the experiments. Author AGN manage the analysis of the study and literature searches. Author DPF wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJI/2018/43510
Editor(s):
(1) Dr. A. Shajahan, Department of Botany, Jamal Mohamed College (Autonomous), Tamil Nadu, India.
Reviewers:
(1) Anna Pick Kiong Ling, International Medical University (IMU), Malaysia.
(2) Shibabrata Pattanayak, Institute of Animal Health & Veterinary Biologicals(R&T), India.
(3) Muhammad Shahzad Aslam, Pakistan.
Complete Peer review History: http://www.sciencedomain.org/review-history/26919

Original Research Article

Received 25 July 2018
Accepted 29 September 2018
Published 29 October 2018

ABSTRACT

Aims: The cosmic production of biomass and bioactive compounds at pilot scale with minimum production costs is an important task to achieve feasible production process of corresponding secondary metabolites at a commercial level.
Materials and Methods: The cell suspension cultures of *Catharanthus roseus* in MS medium supplemented with 2, 4-dichlorophenoxyacetic acid (9.05 µM), kinetin (4.52 µM) were scaled up in a pilot plant bioreactor (100 lit). The cost of production was reduced by addition of substitute carbon source in a basal medium which hardly costs 30% in the medium. Preliminary studies were performed in the 7-lit bioreactor. A 100 lit stainless steel bioreactor equipped with helical impeller top mounted was used for scale-up of *C. roseus* suspension cultures and ajmalicine production.

**Corresponding author: E-mail: dpfulzele@gmail.com;*

Results: The culture medium reduced the cost by 36% by addition of commercial grade sugar whereas medium consist of tissue culture grade sucrose costs 53 USD per 100 lit. The suspension cultures were cultivated in a 100 lit bioreactor containing MS medium fortified with cost-effective carbon source produced ajmalicine 73.18 mg/l DW and achieved 36 kg of fresh biomass on day 20.
Conclusion: The results of the present finding demonstrated the feasible and cost-effective production process of ajmalicine at pilot scale.

Keywords: Bioprocess design; Catharanthus roseus; carbon source; large-scale cultivation; bioreactor; ajmalicine.

1. INTRODUCTION

Catharanthus roseus (L.) G. Don (Apocynaceae), commonly known as Madagascar rosy periwinkle, is a source around 130 indole alkaloids including critical anticancer drugs vinblastine and vincristine are marketed for more than four decades [1]. Ajmalicine antihypertensive drug found as a natural bioactive compound in 20 species of the genus *Rauwolfia*, four of *Catharanthus*, and two of *Mitragyne* [2]. Additionally, particular attention was given to the production of the anti-hypertensive and anti-neuro-inflammatory agent monomeric indole alkaloid ajmalicine which increases the supply of oxygen to cerebral tissues and beneficial in post-stroke rehabilitation [3-4]. Herein, we have focus on the stem part of *C. roseus* because it contains antihypertensive drug ajmalicine, while leaves contain antitumor drugs vinblastine and vincristine [5]. The continuous demand of indole alkaloids in the global market and low yield of alkaloids in field-grown plants has attracted cost-effective tissue culture technology. Since the global market of ajmalicine is gradually increasing and therefore, the large-scale cultivation in the bioreactor is an alternative source for bulk production of life-saving drugs. Statistically, low-cost culture medium reduces the manufacturing cost much lower [6]. The main problem obstructing the development of scale-up of plant cell cultures have been low productivity and the high cost of culture medium. Thus, large-scale cultivation of plant cell culture in a bioreactor with minimal costs of the culture medium is the economically feasible way of producing high-value bioactive compounds from rare and endangered plants.

Previously, remarkable progress for improving the yield of bioactive compounds in the bioreactor was studied extensively under different parameters. Different bioreactor design provides optimal environmental conditions for efficient cell growth and medicinal metabolites production. The scalability and sustainable conditions of a bioreactor which offers an appropriate mass transfer, adequate mixing, and reduced the shear stress intensity to the cultured cells to be promoted for industrial scale and enhance production capacity. However, large-scale cultivation of plant cell cultures in different types of bioreactors for the bulk production of secondary plant metabolites has certain limitations. Over the subsequent 40 years, studies have focused on scale-up of cell cultures in different types of bioreactors to achieve high biomass and product yield. One of the best examples of *in vitro* cell cultures against the field-grown plants produced product is shikonin, in that the extended growing period of 5-6 years entail for the accumulation of metabolites under stable environmental conditions means that the costs of the field-grown raw materials are high. Many researchers have demonstrated the positive approach towards in large-scale applications and economically feasible way of producing high-value plant secondary metabolites [7-12]. These are expected to remain an incredible area in the future for secondary metabolites production in bioreactors at a commercial level.

Although remarkable and attractive advances of plant biotechnology, the production cost of secondary metabolites by cell cultures are still high. Zenk [13] reported that development of plant cell cultures at an industrial scale is preferable if the production cost equal or lower than the field produced the product. A recent study showed that application of disposable bioreactor cut down the production costs for cell culture system for the production of significant secondary metabolites [14-15]. Conversely, plant cell and tissues in the culture medium lack the autotrophic ability and therefore need an external carbon source. Different carbon sources to varying concentrations in culture medium have been utilised to stimulate the product synthesis and reduce the cost of production of secondary metabolites. The optimised protocols have been studied using different carbon source for the

production of withanolide by *Withania somnifera* suspension cultures and achieved 5% more production from a medium enriched with sucrose as a carbon source [16].

A low yield of secondary metabolites by cell cultures with a high cost of culture medium drastically reduced the possibility to make a feasible and viable production process at a commercial level. Furthermore, the supplements of other costly ingredients in MS medium which shares in meager quantity while the source of carbon contributes 83% which increases the costs of the medium. Sucrose remains the most widely used carbon source and is an essential ingredient of culture medium. Due to low productivity and high production costs of these alkaloids by cultures of *C. roseus*, efforts were made to minimise the costs by the addition of commercial grade sugar into the culture medium. There are no reports based on scientific observation in the literature on the scale-up of ajmalicine production at pilot scale. Therefore, in the present study, the culture medium was fortified with low-cost carbon source components for suspension cultures of *C. roseus* in the bioreactors agitated at 100 rpm and 140 rpm and studied the cell growth and ajmalicine production thoroughly.

2. MATERIALS AND METHODS

2.1 Establishment of Callus Cultures

Surface disinfection of explant materials as described by Kapare et al., [17]. In brief, explant materials (stem) of *C. roseus* were kept under running tap water for 30 min and then washed with Dettol for five min followed by thorough washing with distilled water to remove the traces of germicidal agent. The materials were then treated with 70% ethyl alcohol for 3 min and mercuric chloride (0.1% w/v) solution for 2 min, and subsequently, the plant materials were thoroughly rinsed six times with sterilised distilled water under aseptic condition. Lastly, surface sterilised explants were incised into small pieces (10-12 mm) and transferred to Murashige and Skoog's (MS) [18] medium supplemented with 2, 4-dichlorophenoxyacetic acid (9.05 µM), kinetin (4.52 µM) and 3% tissue culture grade sucrose. The medium pH was adjusted to 5.8±0.02 using 0.1N HCl or 0.1N NaOH before addition of agar (8 g/l). Medium (20 ml) dispensed into test tubes and autoclaved at 103.42 kPa for 20 min. Surface sterilised explants were aseptically transferred into culture tubes and incubated at 25±2°C and maintained 16 h photoperiod with white fluorescent light (40 $\mu molm^{-2}s^{-1}$). The cultures were regularly sub-cultured every third week on the same medium composition and kept under similar culture conditions.

2.2 Establishment of Suspension Cultures and Scale up

Suspension cultures were initiated from callus cultures. After 7th subcultures, ~3-4 g of biomass was transferred into 250 ml Erlenmeyer flask containing 75 ml of MS medium fortified with 2, 4-dichlorophenoxyacetic acid (9.05 µM), kinetin (4.52 µM) and 3% tissue culture grade (TCG) sucrose (Hi-media, India). The initial Packed Cell Volume (PCV) of 15% and initial biomass to liquid ratio of 40 g/l FW was achieved from 3-4 g of biomass as an inoculum. Similar attempts have also been carried out with MS medium fortified with the same growth regulators with the addition of 3% commercial grade (CG) sugar (local market, Indore, India). The flasks were kept on Gyratory shaker and agitated at 120 rpm at 25±2°C under 16/8 h light and dark photoperiod conditions with white fluorescent light (40 $\mu molm^{-2}s^{-1}$). The suspension cultures were subculture after every three-week interval.

Firstly, scale-up of suspension cultures was carried out in 1-l Erlenmeyer flasks containing same media constitute and subsequently scaled up in 5-l Erlenmeyer flasks and kept on Gyratory shaker at 120 rpm. For 100-lit bioreactor, eight-nine 5-l Erlenmeyer flasks were used to produce inoculum. In 5 lit Erlenmeyer flasks, fifteen percent packed cell volume was achieved by transferring ~60-70 g fresh biomass of three-week-old suspension cultures in 1500 ml MS medium. The flasks were agitated on Gyratory shaker at 120 rpm at 25±2ºC. After three-week spent medium was removed from each flask and cells were transferred aseptically into 10 lit sterilised inoculum bottles, as inoculum for a 100 lit bioreactor.

2.3 Configuration of 7 lit Bioreactor

The working volume of 7 lit stirrer bioreactor was 4.5 lit, and schematic diagram of 7 lit bioreactor shown in Fig. 1. Top driven agitator fixed on the top, and upper stainless steel top plate was removable. Suspension cultures were cultivated in a bioreactor at 100 rpm and 140 rpm. An internal diameter of bioreactor was 135 mm and impeller diameter 90 mm, length 200 mm and shaft length 360 mm. Total six numbers of

impellers were fixed to impeller shaft and distance between two impellers 40 mm and width of impeller 90 mm. Suspension cultures were aerated by sterile air, supplied by stainless steel orifice at the bottom of the helical impeller. The temperature was maintained at 25±0.5°C. Samples were withdrawn every fifth day for determination of growth and ajmalicine contents. All experiments, data were from the duplicate and the averages with standard deviations given in the results.

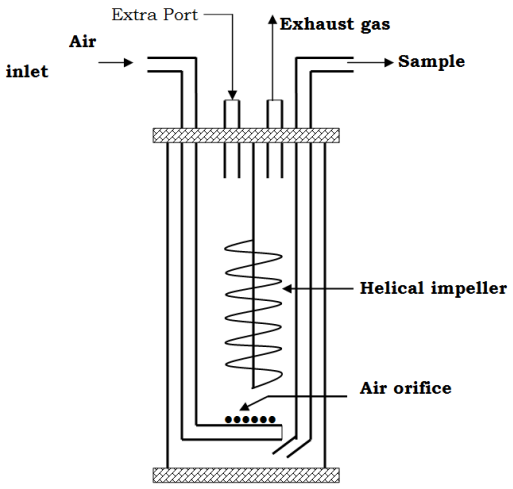

Fig. 1. Schematic diagram of a 7 lit stirrer bioreactor equipped with top mounted helical impeller as agitator. The working volume of bioreactor was 4.5 lit

2.4 Pilot Scale Bioreactor (100 Lit Capacity)

A 100 lit stainless steel bioreactor with the jacket equipped with helical impeller top mounted was used for scale-up of *C. roseus* suspension cultures and ajmalicine production. The internal diameter of a 100 lit bioreactor was 400 mm and working volume of 75 lit. A schematic diagram of a 100 lit bioreactor is shown in Fig. 2. A mechanically top driven helical impeller achieved agitation speed 100 rpm and 140 rpm. The impeller diameter, the diameter of the shaft and length of the shaft were 536 mm, 40 mm, and 660 mm, respectively. The impeller thickness of 5 mm, the total number of impellers 6, the distance between two impellers 76 mm and width of impeller 260 mm. Bioreactor installed with a thermocouple, sterilisable pH and DO electrodes (Ingold, Germany) was used to control temperature, pH and dissolved oxygen of the culture medium. The temperature of suspension cultures in the bioreactor was maintained at 25± 0.5°C in each experiment. The pH of the medium was maintained between 5.7 and 5.8 by the controlled addition of 0.2% NaOH or 0.2% HCl solution in each experiment. Compressed air aerated suspension cultures and filtered by passing through the primary filter to remove oil and particulates and after that air sterilised by passing through 0.22 mm filters (Domnick Hunter) and aeration (0.33 M_3/h) provided via a stainless steel ring positioned beneath the helical impeller. Dissolved oxygen in the medium was maintained at 30% ± 1% from air saturation by a continuous feed of sterile air.

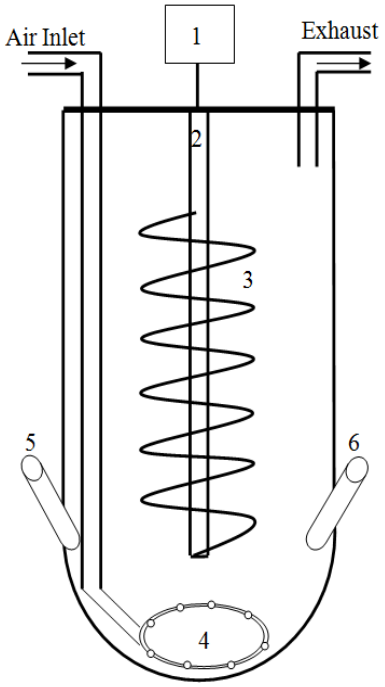

Fig. 2. Schematic diagram of a 100 lit stainless steel bioreactor with the jacket equipped with top mounted helical impeller. The working volume of bioreactor was 75 lit. 1. Stirrer motor, 2. Shaft, 3. Helical impeller, 4. Air orifice, 5. Dissolve Oxygen probe, and 6. Temperature probe

A 100 lit bioreactor was agitated at 140 rpm containing three-week-old suspension cultures to achieve initial biomass to liquid ratio of ~40 g/l FW. The bioreactor comprised 75 lit MS medium supplemented with TC sucrose and CG sugar as a carbon source and experiments were conducted at 100 and 140 rpm individually. Samples (~200 ml) were withdrawn aseptically

from bioreactor every 5th day for determination of pH, fresh weight, dry weight, sucrose, and ajmalicine content. All experiments, data were from the duplicate and the averages with standard deviations given in the results.

2.5 Extraction of Ajmalicine

The extraction of ajmalicine from biomass as described by Namdeo et al., [19]. In brief, with modification, harvested biomass and medium were analysed for the determination of ajmalicine. Suspension cultures were separated by filtration (Whatman No. 41 filter paper). Subsequently, harvested cells were dried in an oven at 55°C and after that powdered. Dried cells were extracted with methanol (three times) and evaporated to dryness at 40°C *in vacuo* to yield the residue. For analysis, the concentrate was transferred into polypropylene micro-centrifuge tubes, mixed with methanol, and centrifuged at 12000xg for 10 min. After centrifugation, clear supernatants were transferred to clean glass vials and directly applied onto a high-performance liquid chromatography (HPLC) column. Similarly, the spent medium was extracted with chloroform, which was separated and evaporated to obtain a concentrate. For HPLC the concentrate was treated as above.

2.6 High-Performance Liquid Chromatography (HPLC)

Quantification of ajmalicine as described by Namdeo et al., [19]. With modification, the HPLC was performed on Shimadzu HPLC system, Japan, (LC-10AT/SPD-10A, CR6A) with a 25 ml loop and a variable wavelength detector. Separations were performed on Inertsil C18 reverse phase silica gel column (4.6 x 250 mm Sigma, USA). The ajmalicine was determined by using Methanol - 0.1 mM di-ammonium hydrogen phosphate (40:60) as a mobile phase. The flow rate was 1 ml/min, and the elution was monitored at 254 nm. This method is sensitive and accurate with good reproducibility. Validation of quantitative process was performed with samples for five times. The results of the five injections from the same samples at five concentrations (0.01–0.5 µg) showed similar retention time. The analytical operation can be completed in 20 min. Quantitative estimation of ajmalicine was performed by using a standard curve obtained from the standard sample (Sigma). Also, Peak identification was confirmed by standard sample of ajmalicine.

2.7 Growth Parameters

Harvest biomass from bioreactor every 5th day was taken up for the determination of packed cell volume (PCV), sucrose, fresh weight (FW) and dry weight (DW). Details on the determination of FW, and DW, were described by Fulzele and Heble [8]. In brief, PCV was determined by allowing 100 ml cell suspension to settle in a 100-ml cylinder for 20 min. Subsequently, harvested biomass was gently pressed on filter papers (Whatman No. 1) to remove excess water for FW determination. Harvested biomass was dried in an oven at 55°C for 16 h to get DW. The determination of sucrose as described by Fulzele and Heble [8], In brief, the measurements of the refractive index have been performed on Bausch and Lomb, USA. The results of the samples of TCG and CG at the different concentrations (0.5% to 5.0%) showed a similar refractive index. Validation of quantitative method was performed with five times the samples. This method is accurate with good reproducibility.

2.8 Statistical Analysis

The influence of carbon source on cell growth and ajmalicine production were determined by one-way analysis of variance (ANOVA). All experiments were carried out in duplicates in bioreactor and data were expressed as a mean ± standard deviation.

3. RESULTS AND DISCUSSION

Large-scale cultivation of suspension cultures in the bioreactor is an attractive bioprocess tool to develop feasible production protocol of secondary plant metabolites. The suspension cultures have been successfully cultivated in the bioreactors and studied the production protocols of alkaloids, isoflavones, terpenoids, taxane, anthocyanin, and glycosides [7-8,20-22]. However, the production costs of the plant secondary products are always a critical task to make feasible and economical production at large scale. It recorded that sole source of carbon alone in the culture medium shared 83%. Hitherto investigation of the influence of low-cost carbon source to reduce the production costs and scale up of *C. roseus* suspension cultures in a pilot plant bioreactor has not been studied earlier. In the present study, we have successfully cultivated suspension cultures of *C. roseus* at the pilot plant level and subsequently investigated the low-cost carbon source to reduce the production cost of ajmalicine.

3.1 Effect of Different Carbon Sources on Cell Growth and Ajmalicine Production in a 7 Lit Stirrer Bioreactor

A preliminary study of the effects of different carbon sources on growth and ajmalicine production was conducted in a 7 lit stirrer bioreactor. The time course of growth and ajmalicine production of *C. roseus* cell suspension cultures were grown in MS medium containing TCG sucrose and CG sugar and subsequently scaled up in a 7 lit bioreactor agitated at 100 rpm (Fig. 3 a & b). As can be seen, cell growth revealed an initial lag phase from days 0 to 5, and after that long exponential phase from days 10 to 25 and lastly cells started lysis and showed decline phase till further cultivation. The cells consumed the maximum sucrose during the exponential growth phase, and depletion of sucrose was rapidly after that. The ajmalicine production was not detected during the first ten days and gradually the production after that steadily increased from 10 to 30 days. The suspension cultures were grown in MS medium containing TCG sucrose produced the maximum amount of ajmalicine 19.45 mg/l DW and the biomass 18.34 g/l DW on day 30 (Fig. 3a). However, incremental increase of indole alkaloid production occurred during the deceleration phase when suspension cultures were cultivated with CG sucrose in medium and cells accumulated 21.73 mg/l ajmalicine (Fig. 3b). In this study, higher ajmalicine production occurred during the deceleration phase, and the available carbohydrate source was consumed entirely by the cells.

Furthermore, suspension cultures were cultivated in a bioreactor and increased the agitation speed from 100 pm to 140 rpm. The kinetic profile of cell growth and ajmalicine production of *C. roseus* suspension cultures in a 7 lit bioreactor at 140 rpm and medium comprised of TCG sucrose and CG sugar depicted in Fig. 4 a & b. The growth and ajmalicine production varied when suspension cultures were cultivated at 140 rpm in a 7 lit bioreactor. The initial lag phase was observed for five days and then increased the cell growth till 30 days. The ajmalicine production was not detected during 0 to 10 days, and after that, the production increased gradually until 30 days of cultivation bioreactor. Maximum production of indole alkaloid ajmalicine was obtained during deceleration phase, and the cells consumed sucrose completely. At 140 rpm, cells were grown homogenously in MS medium containing TCG sucrose and obtained 23.13 g/l DW biomass and maximum ajmalicine production of 37.67 mg/l DW on day 30 (Fig. 4a).

The agitation speed of the helical impeller, at 140 rpm favored homogenous cell growth and did not allow cells to settle at bottom bioreactor. Also, maximum cell growth and ajmalicine production achieved during deceleration phase when suspension cultures were comprised of CG as a sole carbon source. The maximum cell dry weight was obtained by cell cultures grown in MS medium with CG on day 25. In the present results, the agitation speed 140 rpm stimulus homogenous cell growth and achieved a maximum 21.25 g/l DW and produced ajmalicine 38.45 mg/l DW on day 25 (Fig. 4b). The present results revealed that the cells were grown homogenously in medium containing CG sugar and agitation speed 140 rpm could not be caused any damage to the cell. As a corollary, these finding suggested that agitation speed of helical impeller at 140 rpm found to be better for growth and production of ajmalicine. Also, the present results indicated that *C. roseus* cells grown uniformly in medium augmented with low-cost carbon source and also produced more concentrations of ajmalicine than that of cells cultivated with commonly used TCG sucrose.

The TCG sucrose has been used as a common carbon source and replaced with CG sugar resulted in a low-cost medium for a component for rapid mass multiplication of *Picrorhiza kurroa* shoot cultures [23]. Our results are in agreement with Lungaho et al., [24] reported that using low-cost CG as a replacement for high-cost TCG sucrose as the source of carbon and obtained better results of *in vitro* cell cultures of *Solanum tuberosum*. The promising results of inexpensive carbon source that enhanced the production of ajmalicine in a 7 lit bioreactor at 140 rpm, we have scaled up suspension cultures in a 100 lit bioreactor and further experiments at 140 rpm.

3.2 Effect of Different Carbon Sources on Cell Growth and Ajmalicine Production in a 100 Lit Stirrer Bioreactor

In pilot plant study, Fig. 5 a & b presents the profile of growth, sucrose consumption, and ajmalicine production during the cultivation under a 100 lit bioreactor agitated at 140 rpm. Three-week-old suspension cultures were transferred into a bioreactor containing MS medium comprised with TCG sucrose and achieved initial

biomass to liquid ratio 0.04 g/ml FW. The cell growth displayed an initial lag phase for first 0 to 5 days, long exponential growth phase between 10 to 25 days and then growth slowing down from days 25 to 30 (Fig. 5a). The cells consumed sucrose almost wholly before reaching to late exponential phase, and maximum biomass and ajmalicine production were achieved during deceleration phase. A maximum ajmalicine production of 40.15 mg/l DW was obtained on day 25 and decrease after that to 39.81 mg/l DW on day 30.

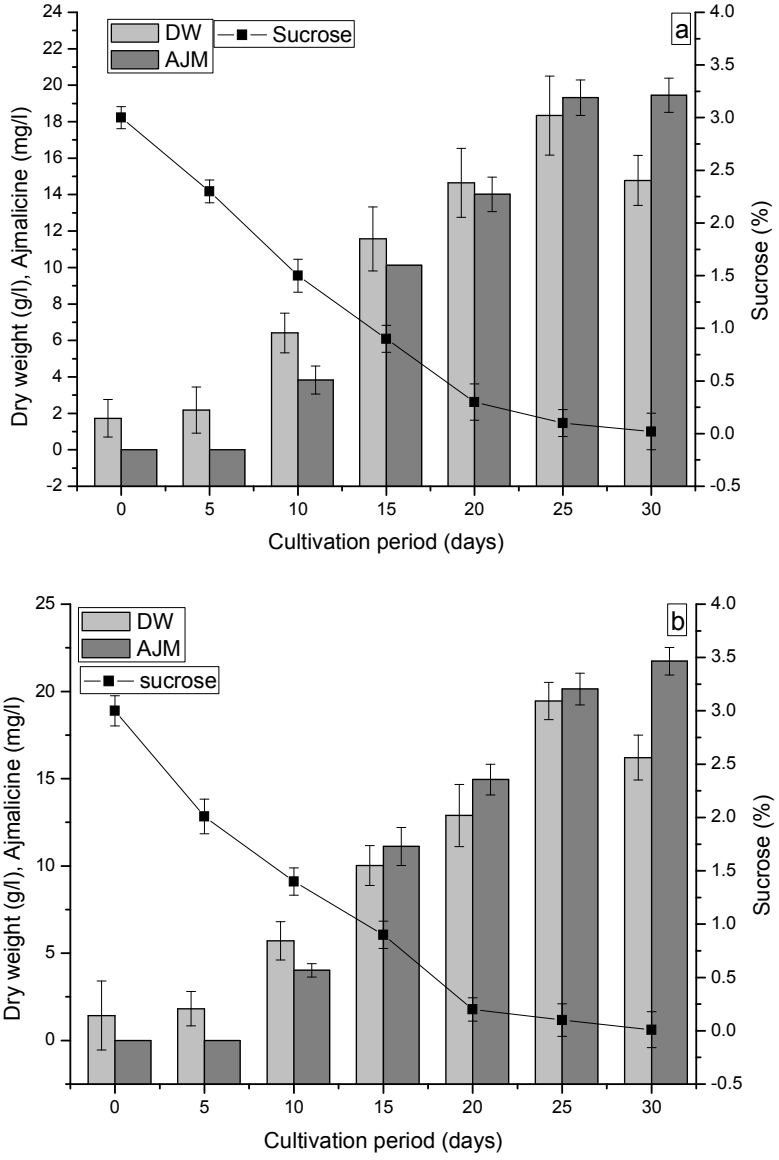

Fig. 3. (a) Time-profile of growth and ajmalicine production by *Catharanthus roseus* suspension cultures in MS medium supplemented with growth regulators and 3% tissue culture grade (TCG) sucrose as carbohydrate source in a 7 lit bioreactor at 100 rpm. (b) Time-profile of growth and ajmalicine production by *Catharanthus roseus* suspension cultures in MS medium supplemented with growth regulators and 3% commercial grade (CG) sugar as carbohydrate source in a 7 lit bioreactor at 100 rpm. These results represent mean values ± SE of two replicates

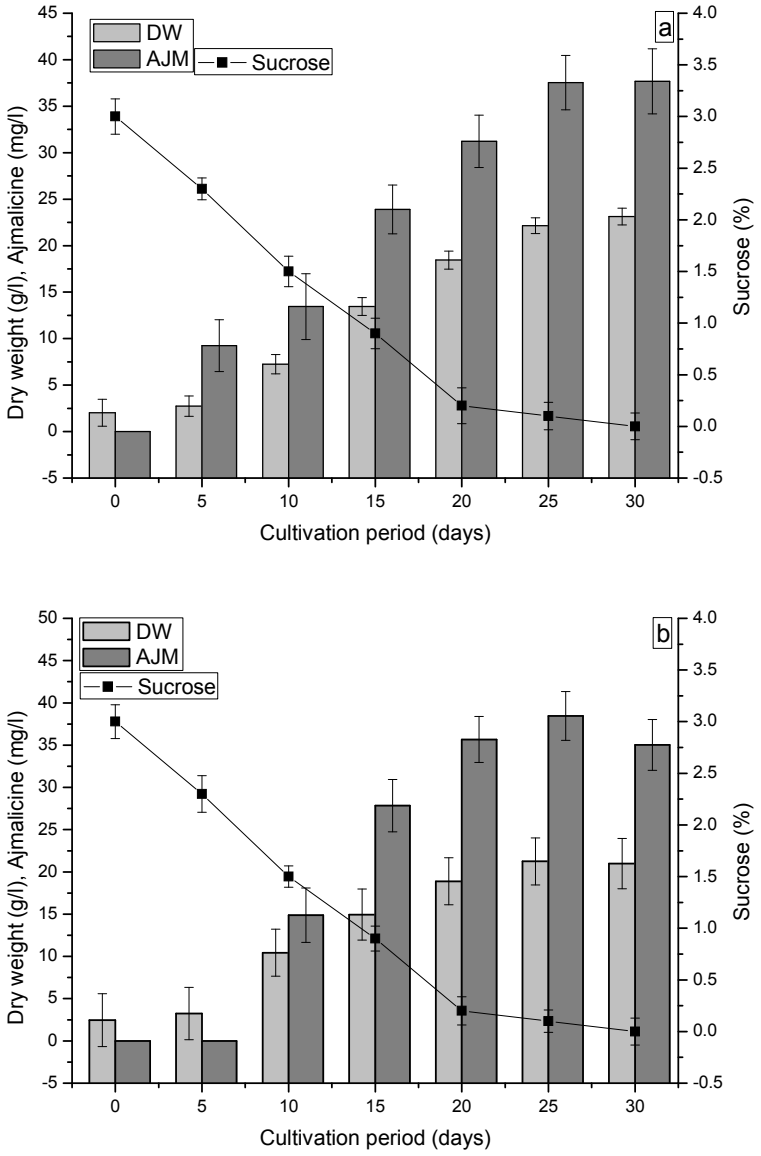

Fig. 4. (a) Time-profile of growth and ajmalicine production by *Catharanthus roseus* suspension cultures in MS medium supplemented with growth regulators and 3% tissue culture grade (TCG) sucrose as carbohydrate source in a 7 lit bioreactor at 140 rpm. (b) Time-profile of growth and ajmalicine production by *Catharanthus roseus* suspension cultures in MS medium supplemented with growth regulators and 3% commercial grade (CG) sugar as carbohydrate source in a 7 lit bioreactor at 140 rpm. These results represent mean values ± SE of two replicates

As well, a low-cost ajmalicine production protocol was studied in pilot scale bioreactor at 140 rpm containing CG sugar as a sole source of carbon. The time course of growth, ajmalicine production, and sucrose consumption is shown in Fig. 5b. The suspension cultures were grown homogenously in a bioreactor, and the cell growth exhibited an initial lag phase for five days, an exponential period between 10 to 20 days and subsequently declined phase. The cells consumed maximum concentrations of sucrose during exponential phase. The ajmalicine production could not detect during the lag phase, but the production level gradually increased from

day ten onwards. The consumption of sucrose rapidly decreased between days 0 to 15 and continued to decline slowly until day 30. The ajmalicine production was augmented during the exponential phase, and at the same time, the cell has rapidly utilised the sucrose in the medium. The maximum ajmalicine production 73.18 mg/l obtained on day 20, and after that, the production was slowly decreased to 62.19 mg/l on day 30 (Fig. 5b). The experimental data exhibited that suspension cultures were acclimatised in a medium comprised of CG sugar and attained 24.28 g/l/day cell growth in a bioreactor.

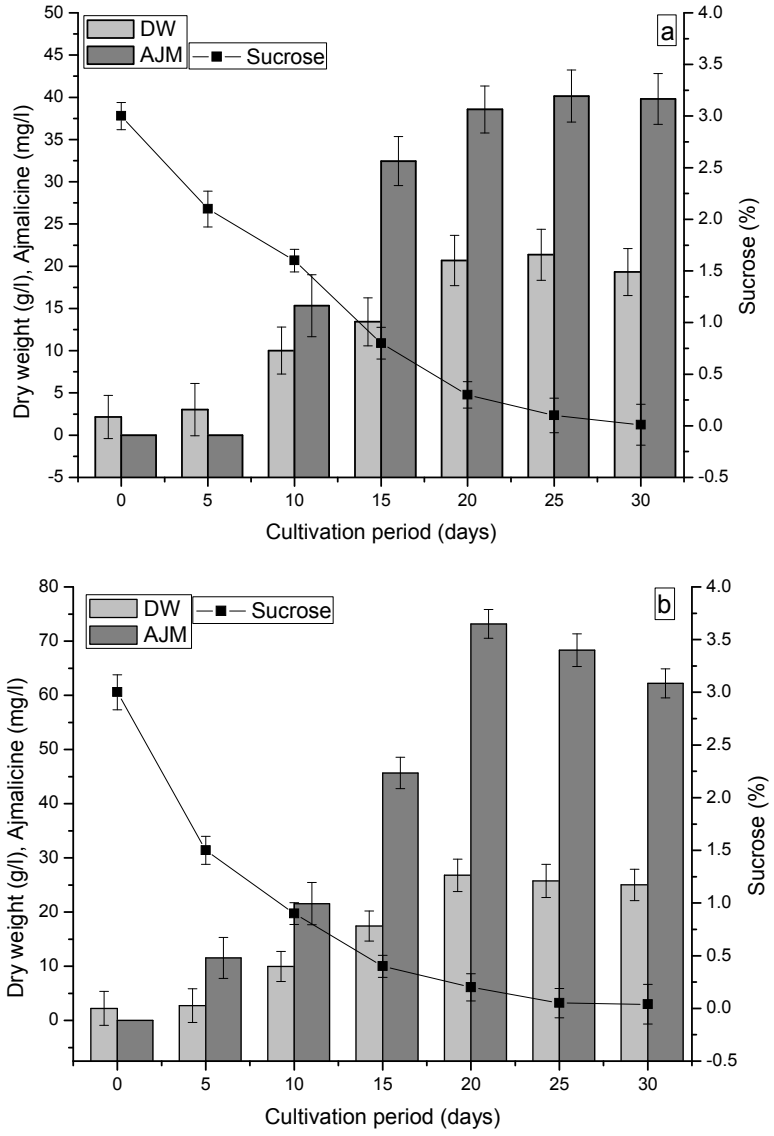

Fig. 5. (a) Time-profile of growth and ajmalicine production by *Catharanthus roseus* suspension cultures in MS medium supplemented with growth regulators and 3% tissue culture grade (TCG) sucrose as carbohydrate source in a 100 lit bioreactor at 140 rpm. (b) Time-profile of growth and ajmalicine production by *Catharanthus roseus* suspension cultures in MS medium supplemented with growth regulators and 3% commercial grade (CG) sugar as carbohydrate source in a 100 lit bioreactor at 140 rpm. These results represent mean values ± SE of two replicates

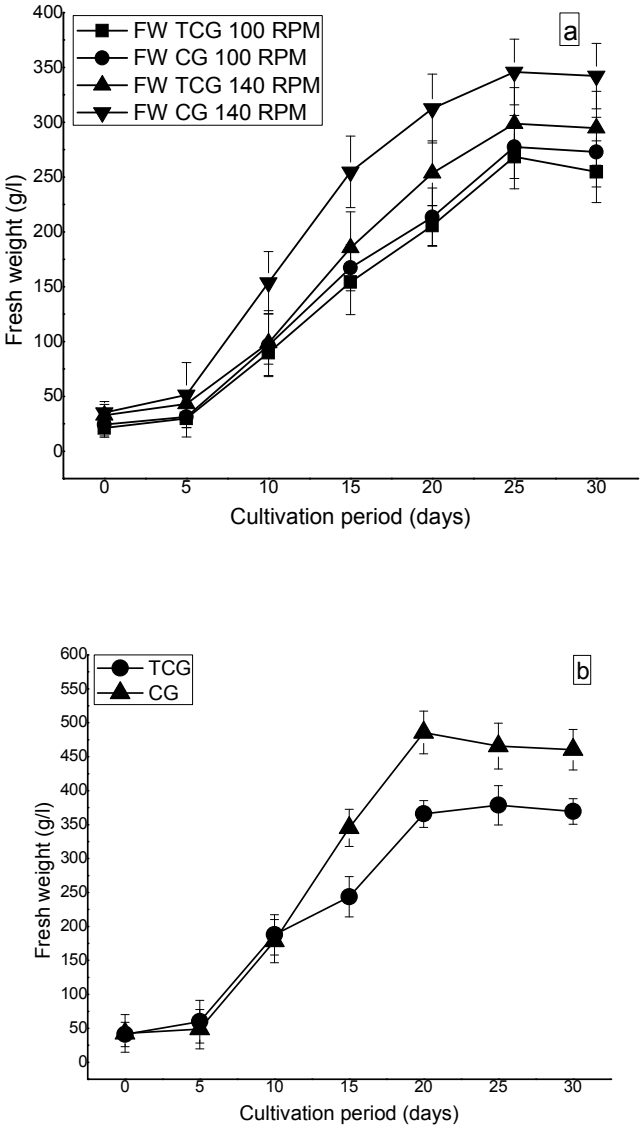

Fig. 6. (a) Time-profile of cell growth by *Catharanthus roseus* suspension cultures in MS medium supplemented with growth regulators and 3% tissue culture grade (TCG) sucrose and commercial grade (CG) sugar as carbohydrate source in the 7 lit bioreactor at 100 rpm & 140 rpm. (b) Time-profile of cell growth by *Catharanthus roseus* suspension cultures in MS medium supplemented with growth regulators and 3% tissue culture grade (TCG) sucrose and commercial grade (CG) sugar as carbohydrate source in the 100 l bioreactor at 140 rpm. These results represent mean values ± SE of two replicates

In this study, the effects of carbon sources on the regulation of ajmalicine production by *C. roseus* suspension cultures were studied in the 100 lit bioreactors, where the inexpensive carbon source CG sugar elevated the ajmalicine production. Growth and ajmalicine production were relatively higher with CG sugar as the carbon source. Suspension cultures were nurtured with TCG sucrose produced ajmalicine 40.15 mg/l DW on 25 days, whereas medium comprised with CG sugar produced 1.8-fold more on 20 days. Further, maximum biomass 26.78

g/L DW and ajmalicine of 73.18 mg/l were output on day 20 when CG sucrose used as a source of carbon in the medium. At the same time, 21.36 g/l DW biomass and 40.15 mg/L ajmalicine produced when medium comprising TCG sucrose, the most commonly used carbon source for plant cell cultures. These findings revealed that the *C. roseus* cells were accustomed to medium containing low-cost CG sugar and attained 3.66 g/l/day ajmalicine. The higher production by suspension cultures of *C. roseus* containing CG sugar conducive for high yield. A 54.86% increase of ajmalicine productivity was enhanced by cultured cells suitable with CG sugar as a carbon source.

We have successfully cultivated suspension cultures in a bioreactor at the pilot plant level and simultaneously studied to reduce the production cost of ajmalicine by the addition of low-cost carbon source. In the present study, the costs of MS medium comprised with TCG sucrose around 53 USD per 100 lit which reduced 36% by addition of low-cost CG sugar, which comes hardly 13 USD per 100 lit. The utilisation of carbon source for product synthesis, the minimal cost of carbon source was found better and stimulated the production capacity of cells and increased the accumulation of ajmalicine in cells (Fig. 5 a & b). These results indicated that low-cost carbon source improved the production level of cultured cells and produced maximum ajmalicine than those grown in MS medium with TCG sucrose. Suspension cultures were grown in MS medium with CG sugar in a 7 lit bioreactor at 140 rpm attained maximum biomass 345.89 g/l FW while cells acclimatised with TCG sucrose produced 298.67 g/l FW (Fig. 6a). The CG grade sugar was found ideal carbon source for the biomass accumulation and product synthesis which yielded 485.65 g/l FW and 73.18 mg/l DW ajmalicine while the culture medium supplemented with TCG sucrose in a 100 lit bioreactor produced 378.56 g/l FW and 40.15 mg/l DW ajmalicine (Fig. 6b). Also, suspension cultures were grown in a 100 lit bioreactor containing CG sugar produced 36 Kg fresh biomass on day 20. Furthermore, the suspension cultures of *C. roseus* were cultivated in the 100 lit bioreactor at 140 rpm produced a high amount of ajmalicine than cells grown in a 7 lit bioreactor at similar agitation speed. A low-cost CG sugar was found suitable like TCG sucrose for cell growth and secondary metabolites production. The present results revealed that *C. roseus* suspension cultures were utilised the inexpensive CG sugar and found to be a better source of carbon for ajmalicine production.

Fig. 7. Pilot-scale ajmalicine production by *Catharanthus roseus* suspension cultures in cost-effective culture medium in the 100 lit bioreactor

Application of low costs medium for plant cell cultures is requisite to reduce the production costs of secondary plant metabolites as the problems associated with slow growth and low product yield. In the present study, we have applied low-cost carbon source to reduce the production cost of ajmalicine at the pilot plant level. The suspension cultures of *C. roseus* cultivated in a bioreactor on two types of carbon sources exhibited differences in their growth and ajmalicine production. We recorded that the cells utilised the low-cost CG sugar which contributes 83% in the culture medium and achieved 55% more production than that of TCG sugar in the culture medium. This results could be due to the

different sensitivity of cultured cells to the 5-hydroxymethylfurfural is an essential bio-sourced intermediate formed during autoclaving from carbohydrates [25]. Also, the incremental increase of cell growth and ajmalicine production was high in cultured cells contained CG sugar may be due to it contains more substances which has already been reported in the regeneration of *Centella asiatica* and *Solanum tuberosum* [26-27]. From the present results, the production of ajmalicine by *C. roseus* suspension cultures in MS medium supplemented with CG was found to be conducive.

The selection of carbon sources which provides energy and beneficial for cell development of *in vitro* cell cultures has long been accepted as a crucial factor for secondary metabolite production by plant cell cultures. Sucrose is found to be most common carbohydrate found in phloem sap. It has been widely used to provide carbon source, energy sources and to maintain osmotic pressure in the cells [28-29]. Also, sucrose recognised as signalling molecules and essential for plant growth and development, stress resistance and secondary metabolites [30-33]. In plant cell cultures, various studies have demonstrated that the appropriate concentrations of sucrose in culture medium enhance the cell growth and also balance the production of secondary metabolites [34-35]. On the contrary, the high concentrations of sucrose in a culture medium that could impede the cell growth and lead to cell death due to increased osmotic pressure [36-37]. Furthermore, sucrose was breakdown to glucose and fructose by cell wall of plant cells [38]. Solfanelli et al., [39] demonstrated that the specificity of sucrose as a signaling molecule and transcript profiling reveals that anthocyanin synthesis pathway in Arabidopsis up-regulated by sucrose. In the present study, the addition of low-cost commercial grade sugar in culture medium has not shown any adverse performance towards cell growth and accumulation of ajmalicine, on the contrary, stimulated the product synthesis. It can be concluded that replacing TCG sucrose which is 36% expensive than that of low-cost CG sugar could be consumed by the cells without any adverse effects on the cell growth and product synthesis.

The CG sugar has been utilised to act as an alternative low-cost medium component for plant cell culture. In the present study, a 100 lit bioreactor operated at 140 rpm, and medium supplemented with a low-cost source of carbon in culture medium achieved better cell growth and ajmalicine production than that of TCG sucrose (Fig. 7). These results are in agreement with Demo et al., (27) reported that low cost CG sugar of local market has more calories and contain high amount of calcium, phosphorous, iron, potassium and sodium compared to TCG. Therefore, additional nutrients present in the CG may further enhance the growth and product synthesis. It has shown that the CG sugar as carbon source was better than the TCG sucrose in various plant cell culture species and achieved rapid multiplication of shoot cultures, micro-tubers and indole alkaloid production [8,40-42]. The present results revealed that a low-cost carbon source in culture medium could be able to reduce the production cost of the bioactive compounds at a commercial level.

4. CONCLUSIONS

The ajmalicine production by *C. roseus* suspension cultures in pilot plant bioreactor was found to be efficient with CG sugar as the medium carbon source which reduced the production cost by 36%. The cell growth and product synthesis were found to be better in CG than that of TCG sucrose which is commonly employed as the carbon source in the plant cell cultures. Furthermore, an agitation speed 140 rpm was found to be appropriate agitation speed for homogenous mixing and does not allow the cell to sediment at bioreactor bottom. Higher amounts of ajmalicine could be obtained in suspension cultures cultivated in MS medium fortified with CG sugar as the carbon source. In a nutshell, it can be concluded that the present findings undoubtedly offer opportunities to improve the production of indole alkaloid by the addition of low-cost carbon source.

ACKNOWLEDGMENT

The authors are thankful to Kabra Drugs Ltd., Indore, India for providing bioreactor facility and activated support of this research work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. van Der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R. The *Catharanthus*

alkaloids: Pharmacognosy and biotechnology. Curr Med Chem. 2004; 11:607-628.

2. Gibbs DR. In: Chemotaxonomy of flowering plants. Montreal-London: McGill-Queen's Univ. 1974;1:214.
3. Li S, Long J, Ma Z, Xu Z, Li J, Zhang Z. Assessment of the therapeutic activity of a combination of almitrine and raubasine on functional rehabilitation following ischaemic stroke. Curr Med Res Opin. 2004;20:409-415.
DOI: 10.1185/030079904125003080
4. Manigandan V, Gurudeeban S, Satyavani K, Ramanathan T. Molecular docking studies of *Rhizophora mucronata* alkaloids against neuroinflammatory marker cyclooxygenase 2. International J Bio Chem. 2014;8:91-99.
5. Yamamoto K, Takahashi K, Mizuno H, Anegawa A, Ishizaki K, Fukaki H, Ohnishi M, Yamazaki M, Masujima T, Mimura T. Cell-specific localization of alkaloids in *Catharanthus roseus* stem tissue measured with Imaging MS and Single-cell MS. Proc Natl Acad Sci USA. 2016; 113:3891–3896.
Available:https://doi.org/10.1073/pnas.1521959113
6. Cui YY, Hahn EJ, Kozai T, Paek KY. Number of air exchanges, sucrose concentration, photosynthetic photon flux, and differences in photoperiod and dark period temperatures affect growth of *Rehmannia glutinosa* plantlets *in vitro*. Plant Cell Tissue Organ Cult. 2000; 62:219–226.
7. Fulzele DP, Kreis W, Reinhard E. Cardenolide biotransformation by cultured *Digitalis lanata* cells: Semi continuous cell growth and production of deacetyllantoside C in a 40-l-stirred tank bioreactor. Planta Medica 58, supple. 1992;7A:601-A602.
8. Fulzele DP, Heble MR. Large-scale cultivation of *Catharanthus roseus* cells: Production of ajmalicine in a 20-l airlift bioreactor. J. Biotech. 1994;35:1-7.
9. Georgiev MI, Weber J, Maciuk A. Bioprocessing of plant cell cultures for mass production of targeted compounds. Appl Microbiol Biotech. 2009;83:809-823. DOI: 10.1007/s00253-009-2049-x
10. Nasim SA, Aslam J, Kapoor R, Khan SA. Secondary metabolites production through biotechnological intervention: Emirates J Food Agricul. 2010;22:147-161.
11. Wilson SA, Roberts SC. Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. Plant Biotechnology J. 2012;10:249-268.
DOI: 10.1111/j.1467-7652.2011.00664.x
12. Alam EA. *In vitro* cultures for the production of some anticancer agents. Life Sci J. 2013;10: 297-310.
13. Zenk MH. Frontiers of plant tissue culture. In: Thorpe, TA. (ed) International association of plant tissue culture. University of Calgary. Calgary; 1978.
14. Terrier B, Courtois D, Henault N, Cuvier A, Bastin M, Aknin A, Dubreuil J, Petiard V. Two new disposable bioreactors for plant cell culture: The wave and undertow bioreactor and the slug bubble bioreactor, Biotech Bioeng. 2007;96:914-923.
DOI: 10.1002/bit.21187
15. Ducos JP, Terrier B, Courtois D. Disposable bioreactors for plant micropropagation and mass plant cell culture. Adv Biochem Eng Biotech. 2009;115:89-115.
DOI: 10.1007/ 10_ 2008_28
16. Sivanandhan G, Kapil Dev G, Jeyaraj M, Rajesh M, Muthuselvam M, Selvaraj N, Manickavasagam M, Ganapathi A. A promising approach on biomass accumulation and withanolides production in cell suspension culture of *Withania somnifera* (L.) Dunal. Protoplasma. 2013;250: 885-898.
17. Kapare V, Satdive R, Fulzele DP, Malpathak N. Impact of gamma irradiation induced variation in cell growth and phytoecdysteroid production in *Sesuvium portulacastrum*. J Plant Growth Regul. 2017;37:919-930.
18. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiologia Plantarum. 1962;15:475–479.
19. Namdeo, A, Patil, S, Fulzele D.P. Influence of fungal elicitors on production of ajmalicine by cell cultures of *Catharanthus roseus*. Biotechnol. Prog. 2002;18:159-162.
20. Kobayashi Y, Akita M, Sakamoto K, Liu H, Shigeoka T, Koyano T, Kawamura M, Furuya T. Large-scale production of anthocyanin by *Aralia cordata* cell suspension cultures Appl Microbio Biotechno. 1993;40:215-218.

21. Pestchanker LJ, Roberts SC, Shuler ML. Kinetics of taxol production and nutrient use in suspension cultures of *Taxus cuspidata* in shake flasks and a Wilson-type bioreactor. Enzyme Microb Technol. 1996;19:256-260.
22. Kokotkiewicz A, Luczkiewicz M, Kowalski W, Badura A, Piekus N, Bucinski A. Isoflavone production in *Cyclopia subternata* Vogel (honeybush) suspension cultures grown in shake flasks and stirred-tank bioreactor. Appl Microbio Biotech. 2013;97:8467-8477.
DOI: 10.1007/ s00253-013-5099-z
23. Sood H, Chauhan RS. Development of a low cost micropropagation technology for an endangered medicinal herb (*Picrorhiza kurroa*) of North-Western Himalayas. J. Plant Sci. 2009;4:21-31
24. Lungaho C, Cheminingwa G, Shibairo S, Hutchinson M, Mbiyu M. Cost effective slow growth *in vitro* conservation of potato (*Solanum tuberosum* L.) using table sugar as an alternative carbon source. African J. Biotech. 2012;11:1092-1099.
25. Lopes de Souza R, Yu H, Rataboul F, Essayem N. 5-Hydroxymethylfurfural (5-HMF) Production from Hexoses: Limits of Heterogeneous Catalysis in Hydrothermal Conditions and Potential of Concentrated Aqueous Organic Acids as Reactive Solvent System. Challenges. 2012;3:212-232.
26. Hossain MA, Hossain MT, Raihan AM, Mahbubur SM. Effect of different carbon sources on *in vitro* regeneration of Indian Pennywart (*Centella asiatica* L.). Pakistan J Biol Sci. 2005;8:963-965.
27. Demo P, Kuria P, Nyende A B, Kahangi EM. Table sugar as an alternative low cost medium component for *in vitro* micro-propagation of potato (*Solanum tuberosum* L.). African J Biotech. 2008;7:2578-2584.
28. Koch K. Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Current Opinion Plant Bio. 2004;7:235–246.
DOI: 10.1016/j.pbi.2004. 03.014
29. Gomez-Ariza J, Campo S, Rufat M, Estopa M, Messeguer J, San Segundo B, Coca M. Sucrose-mediated priming of plant defence responses and broad-spectrum disease resistance by overexpression of the maize pathogenesis-related PRms protein in rice plants. Molecular Plant-Microbe Interactions. 2007;20:832–842.
DOI: 10.1094/MPMI-20-7-0832
30. Wu J, Ho KP. Assessment of various carbon sources and nutrient feeding strategies for *Panax ginseng* cell culture. Appl Biochem Biotechnol. 1999;82:17-26.
31. Birch ANE, Shepherd T, Hancock R, Goszcz K. Understanding sugar sensing in induced plant defences and stress tolerance. In: Proceedings of the 25th meeting of the International Society of Chemical Ecology, Neuchatel, Switzerland; 2009.
32. Moghaddam MRB, Van den Ende W. Sugars and plant innate immunity. J Exp Bot. 2012;63: 3989-3998.
DOI: 10.1093/jxb/ers129
33. Kwon YR, Oh JE, Noh HN, Hong SW, Bhoo SH, Lee HJ. The ethylene signaling pathway has a negative impact on sucrose induced anthocyanin accumulation in Arabidopsis. J Plant Res. 2011;124:193-200.
DOI: 10.1007/s10265-010-0354-1
34. Cui XH, Murthy H, Wu CH, Paek KY. Sucrose-induced osmotic stress affects biomass, metabolite, and antioxidant levels in root suspension cultures of *Hypericum perforatum* L. Plant Cell Tissue Organ Cult. 2010;103:7-14.
35. Ferri M, Righetti L, Tassoni A. Increasing sucrose concentrations promote phenylpropanoid biosynthesis in grapevine cell cultures. J. Plant Physiol. 2011; 168,189–195.
DOI: 10.1016/j.jplph.2010.06.027
36. Al-Khayri JM, Al-Bahrany AM. Callus growth and proline accumulation in response to sorbitol and sucrose-induced osmotic stress in rice. Biol Plant. 2002; 45:609–611.
37. Baque MA, Shin YK, Elshmari T, Lee EJ, Paek KY. Effect of light quality, sucrose and coconut water concentration on the micropropagation of *Calanthe* hybrids ('Bukduseong' × 'Hyesung' and 'Chunkwang' × 'Hyesung'). Aust J Crop Sci. 2011;5:1247-1254.
38. Martinez BC, Park CH. Characteristics of batch suspension cultures of preconditioned *Coleus blumei* cells: sucrose effect. Biotechnol Prog. 1993; 9:97-100.
39. Solfanelli C, Poggi A, Loreti E, Alpi A, Perata P. Sucrose specific induction of the anthocyanin biosynthetic pathway in Arabidopsis. Plant Physiol. 2006;140:637-646.
DOI: 10.1104/pp.105.072579

40. Santana MA, Romay G, Matehus J, Vicente-Villardón JL, Demey JR. A simple and low-cost strategy for micropropagation of cassava (*Manihot esculenta* Crantz). African J Biotech. 2009;8:3789-3797.
41. Sudipta KM, Swamy Kumara M, Anuradha M. Influence of various carbon sources and organic additives on *in vitro* growth and morphogenesis of *Leptadenia reticulata* (Wight & Arn), a valuable medicinal plant of India. Intern J Pharma Sci Rev Res. 2013;21:174-179.
42. Li M, Li J, Liu W, Liu L, Niu J, Liu X, Yang Q. A protocol for *in vitro* production of microtubers in Chinese yam (*Dioscorea opposita*). Biosci Biotech Biochem. 2014; 78:1005-1009.
DOI:10.1080/09168451.2014.912119

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/26919