Bioresource Technology 101 (2010) 6735–6739

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Establishment of cell suspension cultures of *Withania somnifera* for the production of withanolide A

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ARTICLE INFO

Article history:
Received 2 January 2010
Received in revised form 12 March 2010
Accepted 15 March 2010
Available online 3 April 2010

Keywords:
Cell suspension culture
Secondary metabolite
Withania somnifera
Withanolide A

ABSTRACT

Cell suspension cultures of *Withania somnifera* were established in shake flasks and the effect of different growth regulators (auxins, combination of auxin and cytokinin), inoculum density (2.5–20 g L^{-1}), different media (MS, B5, NN and N6), the strength of the MS medium (0.25–2.0×), carbon source (sucrose, glucose, fructose, maltose), concentration of the sucrose (1–8% (w/v)) and the initial pH (4.0–6.5) of the medium were determined for the production of withanolide A. The optimized conditions for biomass accumulation and withanolide A production were found to be 10 g L^{-1} of the inoculum on fresh weight basis, the full strength MS medium, 3% (w/v) sucrose, four weeks culture period and the initial medium pH of 5.8. The results of present study are useful for scale-up process.

1. Introduction

Withania somnifera, also known as ashwagandha, Indian ginseng and winter cherry is an important medicinal plant in Ayurvedic medicine, the traditional medicinal system of India (Gupta and Rana, 2007; Kulkarni and Dhir, 2008). It has been used as a tonic and antistress supplement. Pharmacological activities include physiologic and metabolic restoration, antiarthritic, antiaging, nerve tonic, cognitive function improvement in geriatric states, and recovery from neurodegenerative disorders (Bhattacharaya et al., 2002; Dhuley, 2000). Various alkaloids, withanolides and sitoindosides have been isolated from this plant. Of the various withanolides reported withaferin A and withanone are customary major withanolides of the plant whereas the amount of withanolide A is usually very low (Zhao et al., 2002). Recently, withanolide A has attracted interest due to its strong neuropharmacological properties of promoting outgrowth and synaptic reconstruction (Kuboyama et al., 2005; Tohda et al., 2005a,b). Withanolide A is therefore important candidate for the therapeutic treatment of neurodegenerative diseases, like Alzheimer's disease, Parkinson's disease, convulsions, cognitive function impairment, as it is able to reconstruct neural networks (Tohda et al., 2005a).

In the native plant, withanolide A represents a very minor proportion of withanolide complement and therefore alternative exploration of condition optimized cultures for efficient *in vitro* biogeneration of such withanolides, which are pharmacologically promising but are severely limited in production, is important. Production of withanolide D, withanolide A, withaferin A, withanone have been reported in organogenic cultures including hairy roots (Banerjee et al., 1994; Furmanowa et al., 2001; Murthy et al., 2008; Ray and Jha, 1999, 2001; Ray et al., 1996; Roja and Heble, 1991; Sangwan et al., 2007; Vitali et al., 1996). In the present study we have established cell suspension cultures of *W. somnifera* and examined the effect of various factors on the cell suspension cultures viz. growth regulators concentration and combination, inoculum density, different media (Murashige and Skoog, 1962, MS; Gamborg et al., 1968, B5; Nitsch and Nitsch, 1969, NN; Chu, 1978, N6), the strength of the MS medium, carbon source, concentration of the sucrose, duration of culture period and the initial pH of the medium on the production of withanolide A.

2. Methods

2.1. Plant material and establishment of cell suspension culture

Callus was induced from leaf explants of *W. somnifera* cv. Jawahar on full strength MS (Murashige and Skoog's, 1962) gelled (0.8% agar, w/v) medium supplemented with 30 g L^{-1} sucrose (w/v) and 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg L^{-1} kinetin (KN). Cultures were incubated in the growth chambers at 25 ± 1 °C, with a 16 h photoperiod (40 μmol m^{-2} s^{-1}) provided by 40-W white fluorescent lamps (Philips, Kolkata, India). Cell suspension cultures were initiated by using friable callus in MS liquid medium supplemented with 2.0 mg L^{-1} 2,4-D + 0.5 mg L^{-1} KN in 250 ml Erlenmeyer flasks. The cultures were kept under continu-

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doi:10.1016/j.biortech.2010.03.078

ous agitation at 110 rpm in an orbital shaker (Orbitek, Scigenics, Chennai, India) and incubated at 25 ± 1 °C, with a 16 h photoperiod (40 μmol m^{-2} s^{-1}) provided by 40-W white fluorescent lamps (Philips, Kolkata, India).

2.2. Optimization of culture conditions

Five hundred milligram [fresh weight (FW)] of the cells (the cells were separated from the media by passing them through a 0.45 μm stainless steel sieve under sterile conditions) were cultured in 250 ml Erlenmeyer's flasks containing 50 ml of MS medium supplemented with 3% (w/v) sucrose; the pH of the medium was adjusted to 5.6 ± 0.2 before sterilization by 0.1 N sodium hydroxide (NaOH) and 0.1 N hydrochloric acid (HCl). The cultures were kept under continuous agitation at 110 rpm in an orbital shaker (Orbitek) and incubated at 25 ± 1 °C, with a 16 h photoperiod (40 μmol m^{-2} s^{-1}) provided by 40-W white fluorescent lamps (Philips, Kolkata, India).

The effect of various concentrations (0.1, 0.5, 1.0, 2.0 and 5.0 mg L^{-1}) of auxins [2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA)] and 2 mg L^{-1} 2,4-D in combination with cytokinins [0.1, 0.5, 1.0 and 2.0 mg L^{-1} benzyladenine (BA) and Kinetin (KN)] were assessed on the accumulation of biomass in suspension cultures and the production of withanolide A in an initial experiment. A time-course test was conducted for biomass and withanolide A production and cultures were maintained for 5 weeks, with samples taken at weekly intervals. To determine optimal inoculum density, different levels of inoculants (2.5, 5.0, 10.0, and 20.0 g L^{-1}) were used in separate experiments. In another set of experiments the effect of various media like [Murashige and Skoog (MS), Gamborg's (B5, Gamborg et al., 1968), Nitsch and Nitsch (NN, Nitsch and Nitsch, 1969) and Chu's (N6, Chu, 1978)] and various strengths of MS medium (0.25, 0.50, 0.75, 1.0, 1.50 and 2.0) were tested for the production of biomass and withanolide A. The effect of different carbohydrate sources (sucrose, glucose, fructose, maltose, glucose + fructose (1:1), fructose + sucrose (1:1) and sucrose + glucose (1:1) and the effect of different sucrose concentration (1%, 2%, 3%, 4%, 6% and 8%, w/v) were applied depending on the objective of the experiment. All sugars were filter sterilized using 0.22 μm sterile syringe filters before addition into various media. The effect of different pH [4.0, 4.5, 5.0, 5.5, 5.8, 6.0 and 6.5; pH was set initially before sterilization of the medium by using 0.1 N sodium hydroxide (NaOH) and 0.1 N hydrochloric acid (HCl)] was also assessed for the biomass accumulation and withanolide A production.

2.3. Determination of cell biomass

The cells were separated from the media by passing them through a 0.45 μm stainless steel sieve (Sigma). Dry weights were recorded after the cells were dried at 60 °C till constant weight is recorded.

2.4. Extraction and HPLC analysis

Extraction and HPLC analysis of withanolide A was carried out by following the method of Ganzera et al. (2003). Withanolide A standard was obtained from Chromadex Inc. (Laguna Hills, CA, USA).

2.5. Statistical analysis

All the experiments were set up in a completely randomized design and the data were collected from ten replicates and mean values are subjected to Duncan's multiple range test using SPSS software version 9.0.

3. Results and discussion

There are several reports on accumulation of withaferin A, withanolide D in transformed roots/shooty teratomas cultures, however, the presence of withanolide A has not been detected in those cultures (Banerjee et al., 1994; Ray and Jha, 1999). Recently, Sangwan et al. (2007) have reported presence of withanolide A in the shoot cultures of *W. somnifera* and the levels of withanolide A was matching those in native plants. We explored withanolide A biogenesis in *W. somnifera* cell cultures, and optimized various factors which are essential for the accumulation of withanolide A.

3.1. Effect of different concentration of auxins and or cytokinins on biomass accumulation and withanolide A production

To understand the culture characteristics of the suspended cells of *W. somnifera* in a shake flask, the effect of plant growth regulators [2,4-D or NAA or IBA (0.1, 0.5, 1.0, 2.0 and 5.0 mg L^{-1}) and/or 2.0 mg L^{-1} 2,4-D in combination with BA (0.1, 0.5, 1.0 and 2.0 mg L^{-1}) or kinetin (0.1, 0.5, 1.0 and 2.0 mg L^{-1})] on cell growth and withanolide A production were focused on. The type and concentration of auxin or the auxin/cytokinin ratio alters dramatically both the growth and the product formation in cultured plant cells (Mantell and Smith, 1984). The biomass accumulation and withanolide A content was differing with varied concentration of auxins. The maximum accumulation of biomass (8.11 g L^{-1} DW) and the highest production of withanolide A (1.27 mg g^{-1} DW) was observed in the medium supplemented with 2.0 mg L^{-1} 2,4-D. However, the highest accumulation of biomass (10.79 g L^{-1} DW) and withanolide A (2.26 mg g^{-1} DW) has been observed with the cultures supplemented with 2.0 mg L^{-1} 2,4-D + 0.5 mg L^{-1} KN (Table 1). These results showed that 2,4-D in combination with kinetin was suitable for withanolide A production when compared with all other treatments. Similar to the above results the production of withanolide A in the shoot cultures varied considerably (*ca.* 10-fold, 0.014–0.14 mg g^{-1} fresh weight) according to the phytohormone composition of the culture media as well as genotype used as the explant source (Sangwan et al., 2007). They have reported that 1.0 ppm benzylaminopurine plus 0.5 ppm kinetin was responsible for highest accumulation of withanolide A (14.3 mg per 100 gm fresh weight and 238 mg per 100 gm dry weight, i.e., 0.24%) with the shoot cultures of *W. somnifera.*

3.2. Growth kinetics of W. somnifera cell suspension cultures

Time course accumulation of biomass (dry wt) and production of withanolide A are presented in Fig. 1. The maximum accumula-

Table 1
Withania somnifera cell suspension culture: effect of 2.0 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) in combination with different concentrations of cytokinins on biomass accumulation and withanolide A production.[a,b]

Cytokinins	Concentration (mg L^{-1})	Dry weight (g L^{-1})	Withanolide A content (mg g^{-1} DW)
Benzylaminopurine (BAP)	0.1	5.48 ± 0.06d	1.36 ± 0.02d
	0.5	5.76 ± 0.18d	1.42 ± 0.06d
	1.0	6.51 ± 0.09c	1.82 ± 0.03b
	2.0	6.69 ± 0.06c	1.63 ± 0.02c
Kinetin (KN)	0.1	7.17 ± 0.12b	1.78 ± 0.02b
	0.5	10.79 ± 0.05a	2.26 ± 0.01a
	1.0	6.74 ± 0.16c	1.81 ± 0.06b
	2.0	6.66 ± 0.10c	1.78 ± 0.04b

[a] Cells (0.5 g) were cultured in 50 ml of MS medium for 4 weeks.
[b] Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P \leqslant 0.05$.