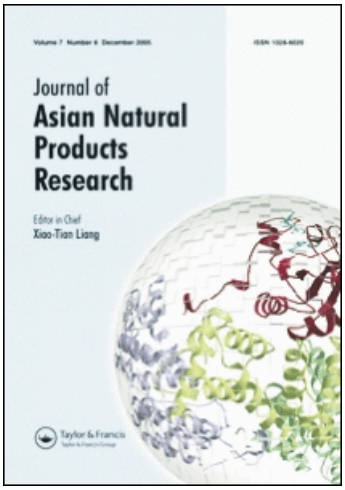

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Biotransformation of cholesterol to diosgenin by freely suspended and immobilised cells of *Dioscorea bulbifera* L.

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Biotransformation of cholesterol to diosgenin by freely suspended and immobilised cells of *Dioscorea bulbifera* L.

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The cell suspension cultures, established from the friable callus which was risen from the nodal segments of *Dioscorea bulbifera* L. in Murashige–Skoog (MS) medium supplemented with indole-3-butryic acid ($20\,mg\,L^{-1}$), was examined for cell growth in MS medium fed with cholesterol ($50\,mg\,L^{-1}$ and $100\,mg\,L^{-1}$) after 8, 10, 12, 14, 16, and 18 days of culture. The growth index of the cell suspension culture on the 8th day was 1.2 and gradually inclined to 1.9 on the 16th day and remained the same at the 18th day. There is no marked difference in the cell growth of cholesterol-treated and control cell suspension culture. The maximum accumulation of diosgenin was noticed on the 14th day in control and cholesterol-treated cell suspension culture and immobilised cell cultures. The highest concentration of diosgenin, 2.94% and 2.14% dry weight, was obtained in immobilised cell culture and cell suspension culture treated with $100\,mg\,L^{-1}$ cholesterol, respectively.

Keywords: Biotransformation; Cholesterol; *Dioscorea bulbifera*; Diosgenin; Growth index; Immobilisation

1. Introduction

Dioscorea bulbifera L. (Dioscoreaceae) is a monocot climber, commonly known as aerial yam, air potato, and bulb-bearing yam, largely distributed in India, Ceylon, the Malay Peninsula, Australia, East Africa and Brazil [1]. It is the only plant of Dioscoreaceae native to both Asia and Africa [2]. *D. bulbifera* is one of the important medicinal plants used in all three indigenous systems of medicine in India [3]. *Dioscorea* species are most noted for the abundance of diosgenin, a steroidal saponin invariably used as a precursor for the synthesis of corticoid steroids, oestrogen, contraceptives, and spiranolactones [4]. Ziagoya *et al.* [5] reported that diosgenin appears to reduce the total body pool of cholesterol, perhaps by blocking intestinal re-uptake of excreted cholesterol. Hypercholesterolaemic rats treated with diosgenin had decreased cholesterol absorption, increased hepatic cholesterol synthesis and biliary cholesterol secretion with no alteration in serum cholesterol [6]. Giving Wistar rats 1% (w/v) diosgenin in their diets increased biliary cholesterol output between 200% and 400% [7,8]. Araghiniknam *et al.* [9] showed that seven elderly human adults treated with up to eight

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wild yam pills daily over six weeks had significant decreases in serum triglycerides and phospholipids, with unchanged serum total cholesterol levels and serum HDL.

Diosgenin is among the ten most important sources of steroids and the most often prescribed medicine of plant origin [10]. The world steroid market has increased linearly at 90×10^3 kg year^{-1} since 1960 and reached 2100×10^3 kg year^{-1} in 1980 [11]. The estimated requirement of diosgenin for the Indian Pharmaceutical industry is around 60 tonnes [12]. In the early 1980s there was a gradual decline in the diosgenin content of yams from 6% to 4% because of over-harvesting, which raised the price of diosgenin from $10 per kg to $25 per kg [13]. Because of the magnitude of the demand and the exorbitant prevailing cost of diosgenin, there is a vigorous search all over the world to meet the demand. In this context an effort is made to enhance diosgenin *in vitro* using biotransformation technology by treating the freely suspended and immobilised cell cultures of *D. bulbifera* with cholesterol.

2. Results and discussion

Friable callus cultures arising from nodal explants of *D. bulbifera* in Murashige and Skoog (MS) medium supplemented with 20 mg L^{-1} indole-3-butryic acid (IBA) was used for the biotransformation studies. Prior to the experiment the viability of the cells was assessed using fluorescein diacetate (FDA). FDA acts as a substrate for the esterase present in the viable cells that will cleave FDA into two components, of which one is fluorescein, which makes viable cells yellow upon excitation [16].

Cell growth was examined in cell suspension culture (CSC) of *D. bulbifera* in MS medium fed with 50 mg L^{-1} and 100 mg L^{-1} cholesterol for 8, 10, 12, 14, 16, and 18 days. The suspension consisted of cell aggregates, in which free cells of diameter ranged from 1 mm to 2 mm and few were marginally more than 2 mm (table 1). The size of the cell aggregates depends on the friability of the callus. The more friable the callus, the smaller the cell aggregates. The growth index (GI) of all the cultures on the 8th day was 1.2 that gradually increased to 1.9 on the 16th day and remained almost the same until the 18th day. It is noteworthy that there is no effect of cholesterol on the cell growth displaying meagre differences between treated and control cultures.

Diosgenin is produced via the mevalonic acid pathway, cholesterol being a key intermediate in its biosynthesis [20]. Diosgenin is synthesised in aerial parts and then translocated to tubers where it is stored [21]. The addition of cholesterol as a precursor to the CSC and immobilised cell cultures (ICC) of *D. bulbifera* has a distinct contribution in the production of diosgenin as compared to cultures without precursor. A similar positive effect of cholesterol addition on diosgenin production in *Trigonella foenum-graecum* suspension cultures was reported [22]. A labelled cholesterol molecule added to *D. deltoideae* cell suspension was found to be wholly incorporated into diosgenin [23]. The cholesterol was utilised as an additional source by the cells in the medium. A little precipitation caused by the addition of the cholesterol feeding to the MS medium was reduced by nitriloacetic acid (NTA), a weak chelating agent.

The biotransformation of cholesterol to diosgenin by CSC and ICC of *D. bulbifera* are summarised in table 2. Diosgenin was not detected in the MS medium during the entire course of study, suggesting that all diosgenin was retained within the tissue. The intracellular production of diosgenin was reported in *T. foenum-graecum* [24]. The maximum accumulation of diosgenin was observed on the 14th day in cholesterol-treated CSC and

Table 1. Growth of cells in cell suspension culture of *D. bulbifera* fed with cholesterol.

			8 days culture		*10 days culture*		*12 days culture*		*14 days culture*		*16 days culture*		*18 days culture*	
Initial inoculum of cells (mg/100 ml)	*Cholesterol treatment (mg L^{-1})*	*Aggregate size*	*FW of cells from CSC (mg/100 ml)*	*GI*	*FW of cells from CSC (mg/100 ml)*	*GI*	*FW of cells from CSC (mg/100 ml)*	*GI*	*FW of cells from CSC (mg/100 ml)*	*GI*	*FW of cells from CSC (mg/100 ml)*	*GI*	*FW of cells from CSC (mg/100 ml)*	*GI*
1000	–	+/++	1260.0 (± 5.77)	1.2	1451.6 (± 6.0)	1.4	1611.6 (± 1.66)	1.6	1790.0 (± 5.77)	1.7	1981.6 (± 1.66)	1.9	1981.8 (± 1.22)	1.9
1000	50	++/+++	1265.0 (± 2.88)^{NS}	1.2	1448.3 (± 1.66)^{NS}	1.4	1655.0 (± 2.88)*	1.6	1813.3 (± 3.33)*	1.8	1990.0 (± 2.88)^{NS}	1.9	1992.0 (± 1.68)^{NS}	1.9
1000	100	++/+++	1266.6 (± 3.33)^{NS}	1.2	1445.0 (± 2.88)^{NS}	1.4	1558.3 (± 1.66)**	1.5	1828.3 (± 1.66)**	1.8	1983.3 (± 3.33)^{NS}	1.9	1988.0 (± 2.22)^{NS}	1.9

Approximate diameter of cell aggregates from suspension cultures: +, < 1 mm; ++, 1–2 mm; +++, > 2 mm. GI, growth index; FW, fresh weight. Data represent an average of 5 replicates. Numbers in parentheses indicate ± SE. Significance relative to control reading: $*P < 0.05$, $**P < 0.01$, ^{NS}not significant.

Table 2. Biotransformation of cholesterol to diosgenin by cell suspension culture and immobilised cell cultures of *D. bulbifera*.

		Diosgenin content									
		8 days		*10 days*		*12 days*		*14 days*		*16 days*	
Test sample	*Cholesterol treatment ($mg\,L^{-1}$)*	*mg/culture*	*% dry weight*	*mg/culture*	*% dry weight*	*mg/culture*	*% dry weight*	*mg/culture*	*% dry weight*	*mg/culture*	*% dry weight*
Control CSC	–	15.98 (±0.30)	1.59	16.70 (±0.05)	1.67	16.98 (±0.98)	1.69	19.30 (±0.03)	1.93	18.37 (±1.66)	1.83
CSC	50	17.46 (±0.04)^{NS}	1.74	17.97 (±0.02)^{NS}	1.79	18.43 (±0.04)^{NS}	1.84	19.87 (±0.01)^{NS}	1.98	18.87 (±0.01)^{NS}	1.88
CSC	100	20.34 (±0.04)*	2.03	20.68 (±0.01)*	2.06	20.92 (±0.01)*	2.09	21.43 (±0.06)^{NS}	2.09	20.45 (±0.03)^{NS}	2.04
Control ICC	–	16.12 (±0.14)	1.61	16.82 (±0.98)	1.68	17.40 (±0.05)	1.74	20.16 (±0.05)	1.74	19.86 (±0.04)	1.98
ICC	50	27.12 (±0.06)**	2.71	27.53 (±0.04)**	2.75	28.10 (±0.05)**	2.81	28.74 (±0.07)**	2.87	28.22 (±0.05)**	2.82
ICC	100	27.96 (±0.06)**	2.79	28.72 (±0.05)**	2.87	28.99 (±0.06)**	2.89	29.48 (±0.13)**	2.94	28.80 (±0.06)**	2.88

Data represent an average of 5 replicates. Numbers in parentheses indicate ±SE. Significance relative to control reading: $*P < 0.05$, $**P < 0.01$; ^{NS}not significant.

ICC of *D. bulbifera* and control cultures as well. The time course for *in vitro* synthesis varies from plant to plant. The diosgenin concentration was highest on the 12th day in *D. deltoideae* [25], and on the 14th day in *D. caucasica* [26] cell suspension cultures, whereas in hairy root culture of *T. foenum-graecum* the diosgenin production increased relatively slowly during the first 20 days to reach maximum after 45 days [24]. The maximum accumulation of diosgenin was noticed on the 14th day in control and cholesterol-treated cell suspension culture and immobilised cell cultures of *D. bulbifera*. The highest concentration of diosgenin, 2.94% and 2.14% dry weight, was obtained in ICC and CSC treated with $100\,mg\,L^{-1}$ cholesterol, respectively. The control (untreated) CSC and ICC produced 1.93% dry weight and 2.01% dry weight of diosgenin, respectively, after 14 days of culture (table 2).

It is evident from the result that $50\,mg\,L^{-1}$ cholesterol treatment increased the diosgenin content from 2.01% to 2.87% dry weight in ICC. However, there was not much increase in diosgenin content in ICC when cholesterol treatment was increased to $100\,mg\,L^{-1}$. The lower concentrations ($10–50\,mg\,L^{-1}$) doubled diosgenin content while at higher concentration this effect was not observed [27]. Diosgenin content started declining after 16 days in all the cell cultures. The accumulation of diosgenin was more in cholesterol-treated ICC than in CSC. Similar results have been obtained during capsaicin production using immobilised cells of *Capsicum frutescens* treated with phenylalanine as a precursor [28].

In spite of steady efforts by various scientists, only a few products such as shikonin, digoxin and ginseng are manufactured on a large scale. However, strategies such as immobilisation coupled with biotransformation indicate the likelihood of many products reaching commercial levels of production. Studies on the purification and characterisation of the enzyme responsible for diosgenin production are in progress.

3. Experimental

3.1 Plant material

Dioscorea bulbifera was collected from the botanic garden, Gulbarga University, and authenticated at the Department of Botany, Gulbarga University with the Flora of Gulbarga District [14] where a voucher specimen is deposited (HGUG (Herbarium Gulbarga University Gulbarga) − 785).

3.2 Callus induction and establishment of CSC (cell suspension culture)

Callus was initiated from the surface-sterilised young nodal segments of *D. bulbifera* in MS (Murashige and Skoog) culture medium [15] supplemented with 3% (w/v) sucrose, $100\,mg\,L^{-1}$ myoinositol, $100\,mg\,L^{-1}$ polyvinylpyrrolidone and $20\,mg\,L^{-1}$ IBA (indole-3-butryic acid). CSC was obtained from the callus tissue (approximately 4*g*) and maintained in 100 ml of MS medium containing $0.1\,mg\,L^{-1}$ 2,4–D (2,4-dichlorophenoxyacetic acid) and $20\,mg\,L^{-1}$ IBA at 120 rpm. Repeated subculturing of callus at every stage obtained fine CSC. Further, the free cells of the suspension were assessed for the GI (growth index) as fresh weight of culture/fresh weight of the inoculum.

3.3 Viability

Viability of cells was determined by the accumulation of FDA (fluorescein diacetate) in the cells [16].

3.4 Immobilisation

Sixteen-day-old cells (1*g*) of cell suspension culture were mixed with 3% (w/v) sodium alginate (Sd Fine Chemical Co., Mumbai, India) in culture medium. Alginate beads were made by dripping the alginate/cell suspension into the medium fortified with 50 mM $CaCl_2$ as previously described by Brodelius *et al.* [17] to obtain ICC (immobilised cell cultures).

3.5 Precursor feeding

Cholesterol (Sd Fine Chemical Co.) was dissolved in hot ethanol (95% v/v), added to 40 ml of MS medium, and supplemented with 100 mg L^{-1} NTA (nitriloacetic acid), 0.1 mg L^{-1} 2,4-D and 20 mg L^{-1} IBA at a concentration of 50 mg L^{-1} and 100 mg L^{-1}, respectively, before autoclaving [18].

3.6 Time course study

Five replicates of cell suspension and immobilised cell cultures treated with cholesterol and control cultures (untreated) were harvested at 8, 10, 12, 14, 16 and 18 days after culture for chemical analysis.

3.7 Chemical analyses

The harvested cultures were separated from the MS medium. The immobilised cells were homogenised in cold methanol and filtered. The residue was refluxed in methanol. The combined solution was evaporated to dryness *in vacuo*. The cell dry biomass were hydrolysed in 1N HCl and extracted with hexane for analysis of diosgenin [19] on a Gas-Chromatograph (Nucon model 5765, New Delhi, India) fitted with a flame ionisation detector at 300°C. A stainless column 10 × 1/8″ packed with Chromosorb AWS (80–100 mesh) coated with 3% SE-30 was employed. The flow rate of N_2 was maintained at the rate of 25 ml min^{-1}. Diosgenin (Sigma Chemical Co., Bangalore, India) was used as a standard reference compound. The amount of diosgenin was determined by comparing the peak areas of the unknown with that of standard solution.

3.8 Statistical analysis

The effect of different treatments was quantified and the data was analysed using a paired *t*-test at 0.01 and 0.05 levels of significance.

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