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Production of Naphthoquinone Pigments in Cell Suspension Cultures of *Arnebia euchroma* (Royle) Johnston: Influence of pH on Growth Kinetics and Acetylshikonin

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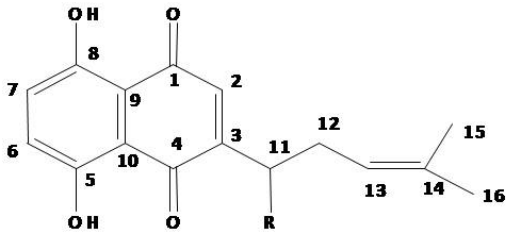
ABSTRACT

In this paper, the effect of pH on *in vitro* cell growth and naphthoquinone pigment production in cell suspension cultures of *Arnebia euchroma* (Royle) Johnston was evaluated under a two-stage culture system. Friable callus derived from leaves of *in vitro* established shoots was maintained on MS medium supplemented with BAP (10 µM) and IBA (5 µM) and the same medium was used for raising cell suspension cultures. An acidic pH (i.e. 5.0-6.50) of the medium helped cell growth, while that having alkaline pH (7.25-9.50) favoured pigment production. Almost double cell biomass (198.67 g, $FW.L^{-1}$) was recorded in medium at physiological pH (5.70), when compared to neutral (138.55 g $FW.L^{-1}$) or alkaline levels (130.56 g $FW.L^{-1}$). Acetylshikonin (I) was found to be the major shikonin derivative in these cultures. The maximum content i.e., 2.049% was recorded at alkaline pH (9.5) in comparison to 0.803% at acidic pH level (5.75) on a fresh weight basis, as revealed by HPLC analysis of the cell suspension cultures. Microscopic examination of the cell suspensions grown at different pH revealed that higher pH adversely affected growth and morphology of the cells. Importantly, the induction of pigment in the alkaline range of pH was independent of the growth phase and pH showed a regulatory role in its biosynthesis.

Keywords: cell biomass, HPLC, plasmolysis, secondary metabolites, shikonin derivatives
Abbreviations: BAP, 6-benzylaminopurine; **FW**, fresh weight; **GR**, growth rate; **IBA**, indole-3-butyric acid; **MS**, Murashige and Skoog (1962) medium

INTRODUCTION

The Himalayan region is a sanctuary for various endangered plant species and *Arnebia euchroma* (Royle) Johnston (family *Boraginaceae*) is one of them. *A. euchroma* commonly known as "Ratanjot", has been traditionally used as a colorant in cosmetics, textile and food industries, besides its application in medicine to treat measles, mild constipation, burns, frostbite, eczema, dermatitis, etc. The roots of *A. euchroma* contain a number of bioactive secondary metabolites including naphthoquinones. Acetylshikonin, β, β-dimethylacrylshikonin, teracrylshikonin, and shikonin or their enantiomer alkannin derivatives (**Fig. 1**) constitute one of the four major naphthoquinone pigments (Ko *et al.* 1995). These are known for a wide spectrum of pharmaceutical properties, including, antimicrobial, anticancer, antipyretic and anti-inflammatory (Kaith *et al.* 1996; Jain *et al.* 2000; Shukla *et al.* 2001; Jiang *et al.* 2005).



Compound	R
(i) Acetylshikonin	$-OCOCH_3$
(ii) β,β-Dimethyacrylshikonin	$-OCOCH=C(CH_3)_2$
(iii) Teracrylshikonin	$-OCOCH_2C(CH_3)=C(CH_3)_2$
(iv) Shikonin	-OH

Fig. 1 Naphthoquinone pigments present in different genera of *Boraginaceae*.

The commercial production of naphthoquinone derivatives has been hampered by the fact that the plant requires three to four years for noticeable production of the pigments. Further, an over reliance on natural sources often puts an undesirable pressure on their sustainability. In this context, the exploitation of cell culture technology for the production of commercially important secondary metabolites offers an attractive proposition. The potential of large-scale production through plant cell culture was realized in the late 1960s (Kaul and Staba 1967; Heble *et al.* 1968). But the low yield associated with cell cultures remained a major bottleneck till the establishment of the world's first commercial industrial unit based on plant cell culture of *Lithospermum erythrorhizon* by Mitsui Petrochemical Industries Ltd. (Japan) in 1983. The success of this industry in the production of shikonin, a dye with anti-inflammatory and antibacterial properties, was based on the sound research and development efforts of scientific and corporate sectors in Germany and Japan (Tabata *et al.* 1976; Zenk *et al.* 1977; Fujita *et al.* 1981; Zieg *et al.* 1983; Yamada 1984; Benjamin *et al.* 1986; Tabata and Fujita 1985). Mitsui Petrochemical Industry Company in Japan was the first to produce shikonin, valued at $4,500/kg, on a commercial scale (Papageorgiou *et al*. 1999). This success prompted investigations into production of such compounds by culturing cells of many other members of *Boraginaceae*. In India, there is scanty information available on R&D work especially with *A. euchroma.* Although some other species such as *A. benthamii*, *A. hispidissima* and *A. nobilis* have been investigated for their medicinal properties, only few reports are available on the establishment of *in vitro* and suspension

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cultures (Shukla *et al.* 1971; Kaith *et al.* 1996; Srivastava *et al.* 1999; Singh *et al.* 2003). *Arnebia euchroma* has also been found to possess high contents of naphthoquinone derivatives including shikonin and therefore, efforts have been made to develop its cell culture (Chen *et al.* 1994; Shen *et al.* 2002).

This paper reports on the production of acetylshikonin in cell suspension cultures, as an alternative production system for these important dyes. Further, this is the first elaborative report on influence of pH on cell growth and production of naphthoquinone pigments in cell suspension cultures of *Arnebia euchroma*.

MATERIALS AND METHODS

Callus and cell suspension cultures

Planting material of *Arnebia euchroma* was procured from KIBBER (*trans* Himalayas; 4200 m above mean sea level). Callus cultures derived from leaves of *in vitro*-grown shoots were maintained on 0.8% (w/v) agar gelled MS medium (Murashige and Skoog 1962) containing 6-benzylaminopurine (BAP 10 µM) + indole-3-butyric acid (IBA 5 µM) + 3% (w/v) sucrose, kept under dark at 25 ± 2°C temperature and sub-cultured every three weeks on to the same medium. Friable callus (two years old) was used for establishing cell suspension cultures in same medium under similar culture conditions.

On the basis of our earlier experiments (unpublished data), we observed that pigment production is not linked to growth, hence a two-stage culture system was adopted i.e. for growth and pigment production. In the case of the growth phase, cells were grown in liquid MS + 10 µM BAP + 5 µM IBA + 3% sucrose (Growth Medium). The cells were inoculated at 10% inoculum density in 100 ml Erlenmeyer conical flask containing 30 ml medium. The suspension cultures were kept under dark at 25°C in an incubated shaker (100 rpm) and maintained with regular sub-culturing at 8-10 days interval.

In the pigment production phase, the cells were inoculated in Plant Growth Regulator (PGR)-free modified White's medium (i.e. APM: Arnebia Pigment Production Medium, **Table 1**). The modification was based on our elaborative studies conducted by altering the type and concentration of various micro- and macro-elements like nitrates, sulphate and vitamins. The inoculum density, volume of culture medium and the culture conditions were the same as described for growth medium.

pH regime

To investigate the effect of pH on cell growth and pigment formation, cells were grown on respective media having varied pH (ranging from 5.00 to 9.50) with an increment of 0.75 units. The pH of media was adjusted with 0.1 N HCl or 0.1 M KOH prior to autoclaving. In both growth and production media, three replicates for each treatment were used and the experiment was repeated thrice using a Complete Randomized Design (CRD).

Table 1 Composition of *Arnebia* Pigment Production Medium (APM).

Constituents	Concentration (mg/l)
$Ca(NO_3)_2 \cdot 4H_2O$	500.00
$MgSO_4 \cdot 7H_2O$	700.00
KNO_3	100.00
KH_2PO_4	170.00
Na_2SO_4	1500.00
H_3BO_3	4.50
$ZnSO_4 \cdot 7H_2O$	3.00
KCl	65.00
$Na_2MoO_4 \cdot 7H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.25
$FeSO_4 \cdot 7H_2O$	27.80
$Na_2EDTA \cdot 7H_2O$	37.30
Sucrose	30000.00

Growth kinetics

Cell biomass

The fresh weight was obtained at 2 days interval. Cell biomass was harvested by sieving suspension cultures through 45 µm mesh and placed the biomass on Whatman 4 filter paper for the removal of excess moisture. The weight was expressed in g, FW/L (gram, Fresh Weight per liter).

Growth rate

Growth rate was calculated on the basis of cell biomass recorded at various intervals using following equation.

Growth rate (GR) = $(W_2 - W_1).t^{-1}.v^{-1}$ = g, FW L^{-1}. D^{-1}

W_1 = Initial weight of cells (g)

W_2 = Final weight of cells (g)

t = Culture period (days)

v = Volume (L)

The cells grown in media having different pH were studied under Nikon Light Microscope (Labophot).

Extraction and quantitative estimation of naphthoquinone pigments in cell culture

Spectrophotometric measurement

For estimation of total pigment content, optical density (OD) of the extracted liquid fraction from suspension was measured at 620 nm. Extraction was based on the procedure described by Yazaki *et al.* (1998). The pigment was extracted from the medium with *iso*-amyl-alcohol, which was converted into free shikonin by an appropriate volume of 2.5% potassium hydroxide (KOH). The absorbance was measured by SPECROD 200, Analytikajena (Germany).

HPLC analysis

Sample preparation

The fresh cells (3.5 g) were extracted with chloroform (3 × 10 ml) under ultrasonication (3 × 10 min). The extracts were filtered and red colour obtained in the chloroform layer, after drying over anhydrous sodium sulphate, was removed under reduced pressure. The yield of extraction was found to be 0.15 ± 0.02% (w/w) on a fresh weight basis in cells. Approximately 10 ml of medium from cell suspension was placed in a 50 ml separating funnel, to which 10 ml of chloroform was added and vigorously shaken. Red colour in the layer containing chloroform was separated and dried over anhydrous sodium sulphate. Chloroform was removed under reduced pressure. The process was repeated thrice. Yield of extraction was found to be 0.05 ± 0.02% (w/v). 1.0 $mg.ml^{-1}$ of each extract (in acetonitrile) was injected into the HPLC column for quantitative determination of acetylshikonin.

Analytical HPLC conditions

HPLC (Waters model 600E system, Waters, Milford, MA, USA) equipped with a reverse phase Purospher®-Star RP-18e column (250 mm × 4.6 mm I.D., 5 µm, Merck, Darmstadt, Germany), a photodiode array detector (Waters 2996), an inline-degasser AF (Waters), a 20 µL loop manual injector and Waters empower software, was used for analysis of naphthoquinone derivatives. Acetonitrile/methanol (95/5, v/v) were used as mobile phase with a 0.6 $ml.min^{-1}$ flow rate in a isocratic elution for 10 min. The column was equilibrated under the starting conditions for 5 min. The detection wavelength was set at 520 nm. The column temperature was 30°C, and the injection volume of samples was 20 µl.

Statistical analyses

Experiments were conducted under a Completely Randomized Block Design (CRD). The experimental data were analysed using 'Statistica' software and significant differences between treatment means were based on Duncan's Multiple range Test.

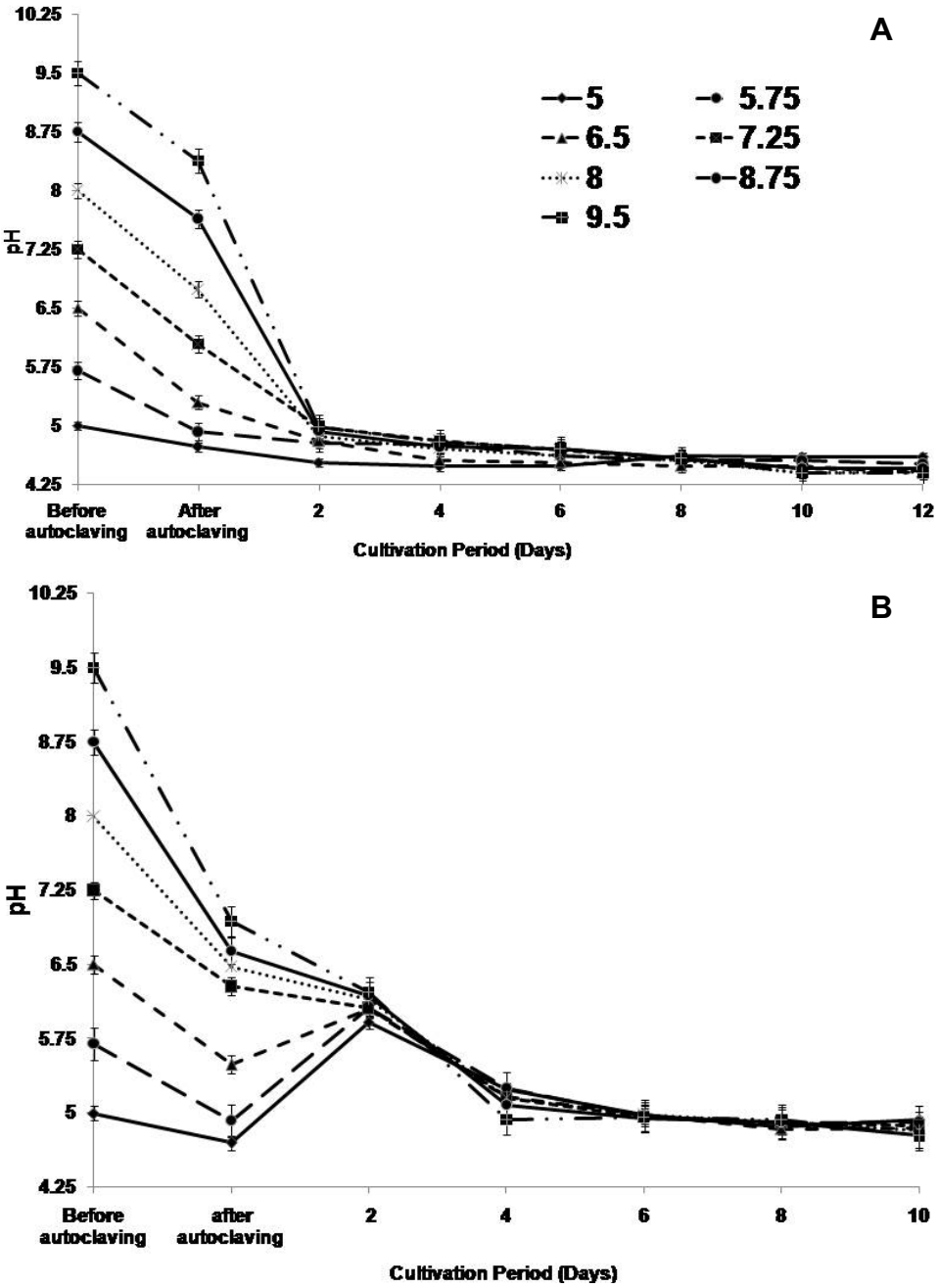

Fig. 2 Changes in pH values of *Arnebia euchroma* cell suspension cultures. (**A**) growth medium and (**B**) pigment production medium (±SD).

RESULTS

Callus induced from leaves of *in vitro* grown shoots of *A. euchroma* on BAP (10 µM) + IBA (5 µM) supplemented MS medium was compact. However, after frequent subculturing, the callus proliferated and became friable. Such calluses were used for raising cell suspension cultures in the same medium. The cell line used in this study was screened from different experiments carried out over a period of two years. The present study demonstrates that pH has an important role in cell growth and production of naphthoquinone pigments in cell suspension cultures.

Changes in pH during cell suspension culture

To investigate the effect of initial pH of the medium in suspension cultures during subsequent growth, pH was recorded at various time intervals (**Fig. 2A**). In the growth medium, there was almost one unit decrease in pH value after autoclaving (**Fig. 2A**) at alkaline pH regime, which was followed by a sharp decline towards the end of second day of culture. A similar trend was observed in acidic pH regime (i.e. 5.00, 5.75 and 6.50), but with a slower rate. After day 2, no significant change was recorded in any of the treatments, as the pH reached a constant value of 4.70-4.90.

The changes observed in APM medium (**Fig. 2B**), revealed a similar but slightly higher initial decrease after autoclaving in case of alkaline pH regime as compared to acidic pH. On day 2, a slight increase in pH of the acidic media (pH 5.00, 5.75 and 6.50) was recorded, however, in alkaline media pH regime continued to decline. The reduction of pH to a level of 5.50-6.50 occurred within 2-4 days of culture. Interestingly, the media was observed to have an acidic pH (4.50-4.90) after day 4 onwards with gradual decline till the end of the culture period.

Effect of pH on cell growth

A positive increase in fresh weight of cell biomass was observed till the eighth day of cell culture. The pattern was almost the same in all treatments; however, there was a lower biomass yield in suspension cultures having alkaline pH (7.25 and above). It is evident from **Fig. 3** that suspension cultures entered into the exponential growth phase on the 4th day of culture, which lasts up to 8 days, before entering into the stationary or declining phase of the cell cycle.

pH 5.75 medium was superior to the rest of the treatments with respect to various growth characteristics. The

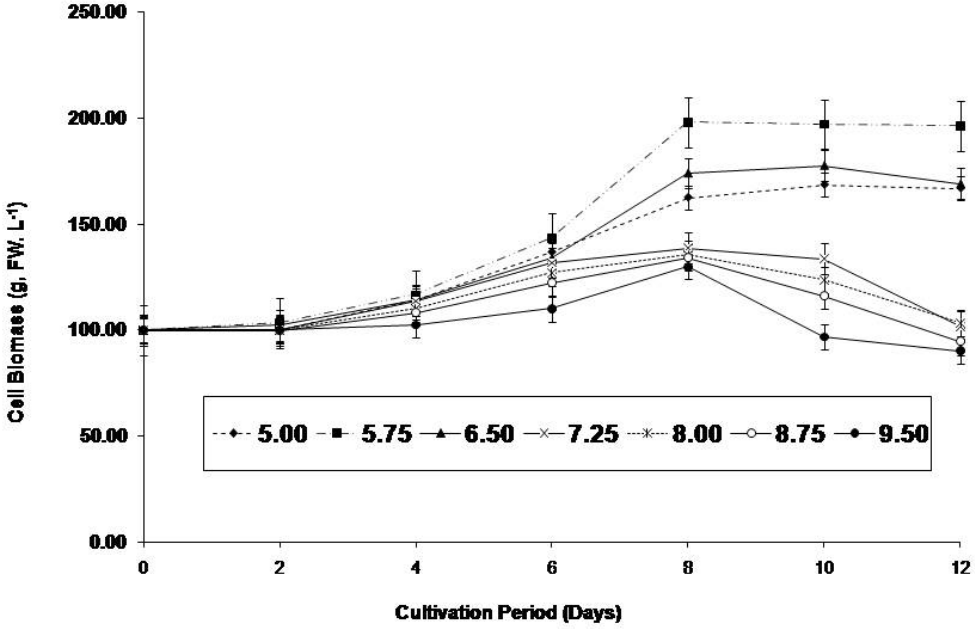

Fig. 3 Effect of pH on cell biomass of *Arnebia euchroma* in cell suspension cultures (±SD).

Table 2 Growth characteristics of *Arnebia euchroma* cell suspension cultures in growth medium.

pH	Cell biomass (g FW.L^{-1})*	Growth rate (g FW.L^{-1} D^{-1})
5.00	162.34 ± 8.11 ab	8.00
5.75	198.67 ± 11.29 a	12.30
6.50	174.10 ± 9.02 ab	10.00
7.25	138.55 ± 6.95 b	4.33
8.00	135.32 ± 6.81 b	3.66
8.75	134.49 ± 6.67 b	3.60
9.50	130.56 ± 6.30 b	2.74

*Mean ± Standard Deviation in triplicate treatments
Different letters within a column indicate significantly different treatment means according to DMRT (P<0.05)

Table 3 Acetylshikonin content in cell suspension cultures of *A. euchroma* at different pH of APM.

pH	Acetylshikonin (%)*	
	Cells	Medium
5.00	0.2986 ± 0.088 d	0.1038 ± 0.008 d
5.75	0.8026 ± 0.056 c	0.2966 ± 0.061 c
6.50	1.0124 ± 0.229 c	0.6420 ± 0.096 b
7.25	0.9446 ± 0.098 c	0.5967 ± 0.016 b
8.00	1.7153 ± 0.171 b	0.5676 ± 0.060 b
8.75	1.7126 ± 0.077 b	0.5544 ± 0.123 b
9.50	2.0498 ± 0.183 a	0.8148 ± 0.113 a

*Mean ± Standard Deviation in triplicate treatments
Different letters within a column indicate significantly different treatment means according to DMRT (P<0.05)

media having a higher pH value (7.25, and above) were observed to have minimum cell biomass and GR (**Table 2**). The cell biomass yield was significantly higher (198.67 g FW L^{-1}) in suspension cultures having pH 5.75 and lowest (130.56 g FW L^{-1}) at pH 9.50. The pattern of cell growth is indicative of 8-10 days sub-culturing period for maximum biomass production.

Effect of pH on acetylshikonin production

A pronounced effect of pH on acetylshikonin production was observed during the time course of cell culture (**Fig. 4**). Cells grown at high pH (9.50) resulted in significantly higher acetylshikonin content (**Table 3**). As the pH lowered from 9.50 to 5.00, its yield also decreased significantly and the period of induction was delayed by almost two days. The maximum content of acetylshikonin (2.049%) was recorded at alkaline pH (9.50) in comparison to 0.2986% at acidic pH level (5.00) on a fresh weight basis, as revealed by HPLC analysis of the cells (**Table 3**). An almost similar trend was recorded in the medium of the suspension cultures (**Table 3**), where significant differences were observed between medium having extreme pHs.

HPLC quantification

The standard acetylshikonin (I, **Fig. 5A**) was extracted and isolated from roots of *A. euchroma* (Shen *et al.* 2002) and its structure was confirmed by NMR (Kirimer *et al.* 1995) The HPLC analysis (Hu *et al.* 2003) of cell culture disclosed the presence of acetylshikonin (I) (R_t = 6.47 min) which was identified by comparison of its retention time, UV-vis spectra and by co-injections with standard I (**Fig. 5A-C**). It was evident from the retention time of the peaks in **Fig. 5B** and **5C** that *A. euchroma* cells grown at pH 5.75 (**Fig. 5B**) and pH 9.50 (**Fig. 5C**) produced similar shikonin derivatives i.e. acetylshikonin.

In growth medium, light microscopic studies revealed that cells cultured at pH 5.75 showed normal growth. As the pH of the medium increased, growth of the cells declined and showed plasmolysis (**Fig. 6**). Highly pigmented and plasmolysed cells were observed at alkaline pH (9.5) of the production medium, whereas at physiological pH i.e. 5.75, cells were healthy and with moderate pigmentation. In growth medium too, a slight pigment formation was noticed at alkaline pH levels (8.0 and above), signifying the role of pH in its biosynthesis.

DISCUSSION

Cell growth and production of shikonin derivatives in cell suspension cultures of *A. euchroma* are influenced by pH of the medium. Results of our experiments showed that pH of both the media changed by 1-2 units (**Fig. 2**) after autoclaving, more so in APM medium. The changes in pH value after autoclaving is understandable, as high temperature alters the composition of various nutrients and salts of the medium (Skirvin *et al.* 1986; George 1993). The initial increase in pH value (**Fig. 2B**) might have resulted from the fast uptake and reduction of nitrates (formation of OH^-) to help in the synthesis of protein for cell growth, while acidification of the medium at later stages of cell culture can be associated with the uptake of ammonium (Fett-Neto and di Cosmo 1996). A further decrease in pH after inoculation (having alkaline or acidic pH) may be attributed to the modifications in cell metabolism for acclimatization to the

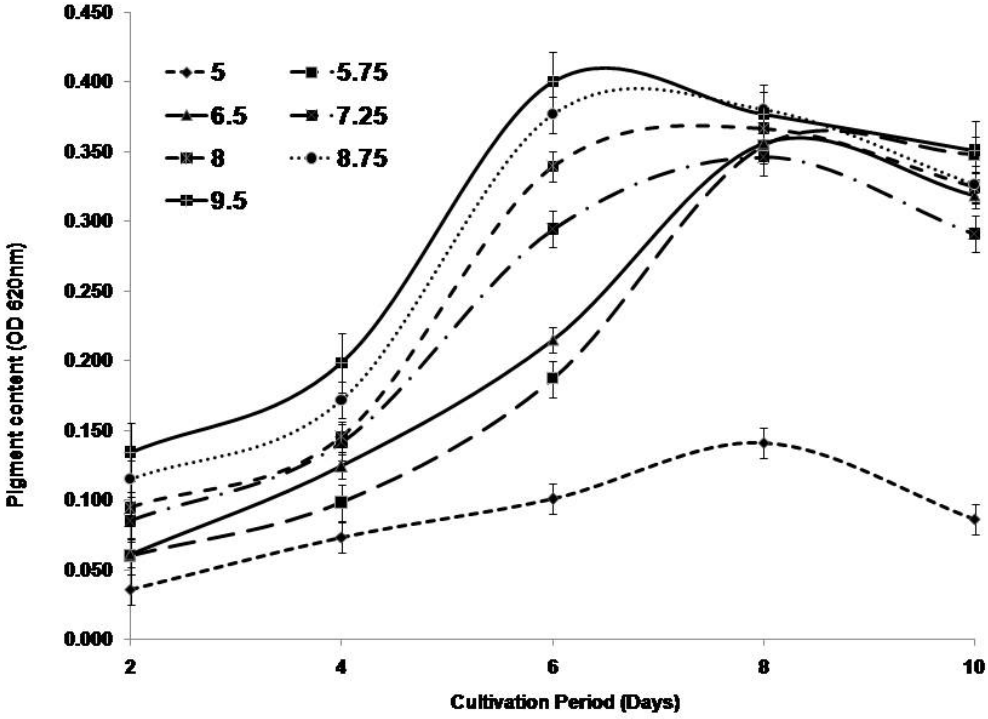

Fig. 4 Effect of pH on pigment production of *Arnebia euchroma* cell suspension cultures (±SD).

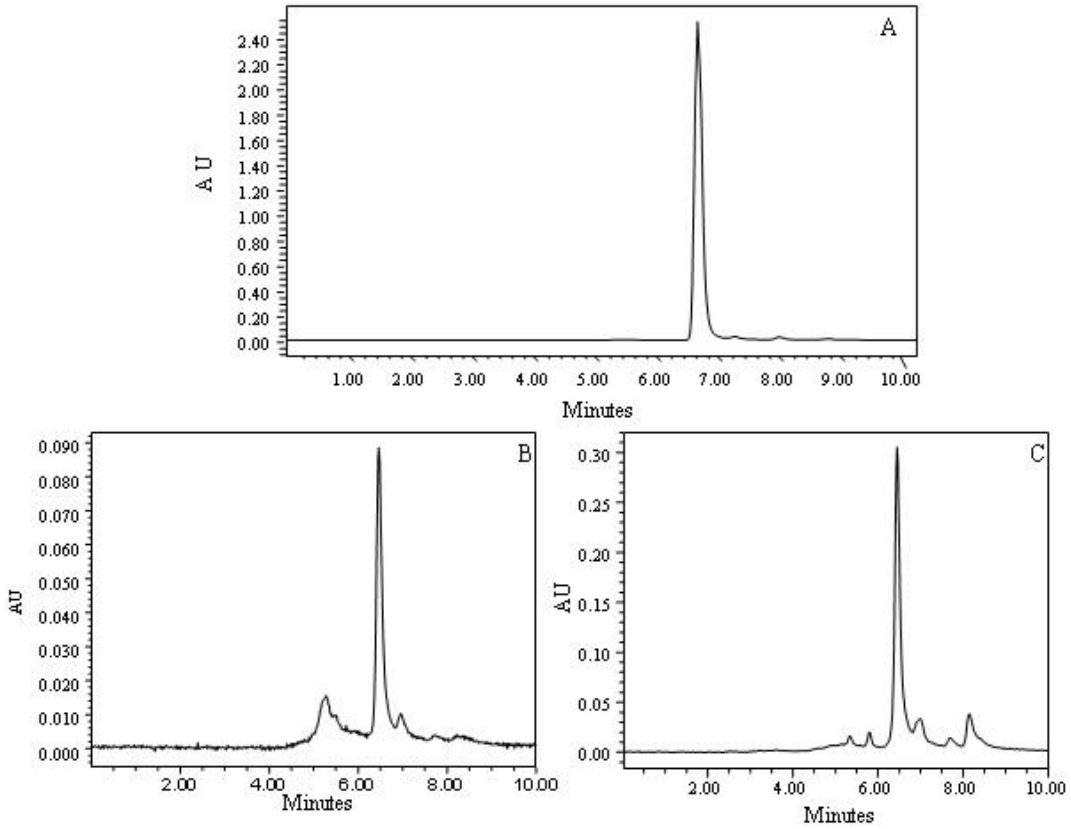

Fig. 5 HPLC Chromatogram of acetylshikonin. (**A**) Standard and (**B, C**) cells from cell suspension cultures of *Arnebia euchroma* grown at pH 5.75 and 9.5, respectively.

new chemical environment, thus resulting in establishment of equilibrium at later stages of cell culture. The electrochemical potential (Δμ) is also a contributory factor for the pH change in medium during cell growth, as it affects the membrane potential (Δψ) (Salisbury and Ross 1992; McCarty 1999). Exposure of cells to extreme low pH leads to conversion of inorganic phosphate into organic phosphate at the extracellular region, which is accompanied by reduction in ATPs thus affecting the cell growth adversely (Mimura *et al.* 2000). The change in pH value (as recorded in APM) can be ascribed to low buffering of the medium, as similar observation was recorded in case of White's medium, which is least buffered (Owen *et al.* 1991).

The maximum biomass yield and high growth rate revealed the superiority of pH 5.75 medium followed by pH 6.50 medium (**Table 2**). It is well established that maximum solubility and absorption of the nutrients in plant cell culture occurs at 5.5-6.0 pH range (physiological pH of plant cells). The fluctuation in pH can alter the growth rate of cells by affecting the transport, mobility and absorption of the nutrients (George *et al.* 1987; Creighton 1999).

Non-growth linked production behavior of the *A. euchroma* cell culture prompted us to employ two-stage culture system. Earlier studies also revealed that the secondary metabolites are produced at the end of the growth phase and under stress conditions (Fujita *et al*. 1982; Scott and Dougall 1987; Yemon 1987; Papageorgiou *et al.* 1999). Therefore, in present investigation, modified WH medium (APM)

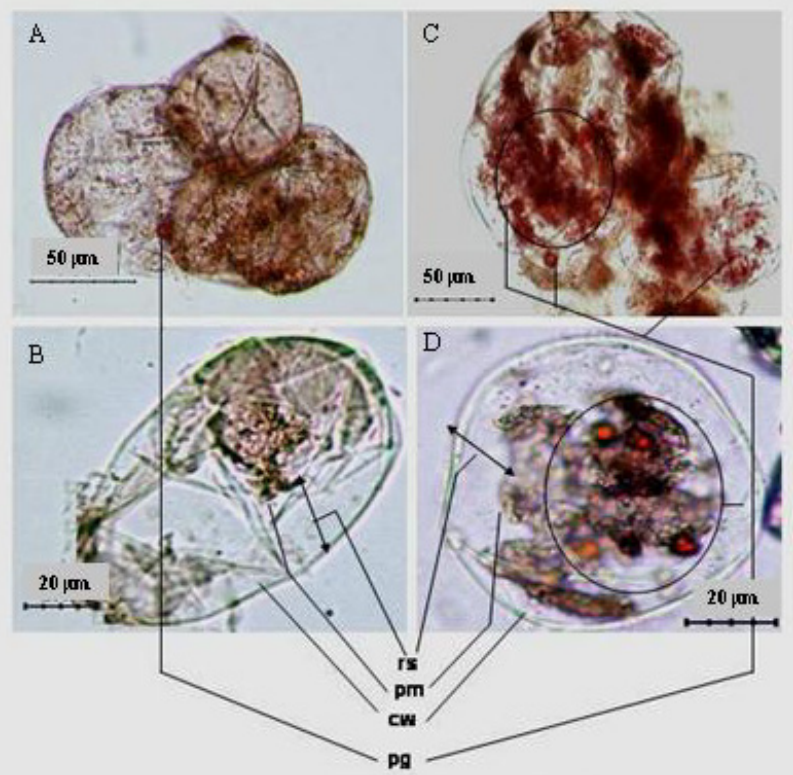

Fig. 6 Microscopic evaluation of *Arnebia euchroma* cells grown at (**A**) pH 5.75 on growth medium; (**B**) 5.75 pH production medium; (**C**) 9.5 pH growth medium; (**D**) 9.5 production medium. rs, retraction space; pm, plasma membrane; cw, cell wall; pg, pigment granules.

having low salt concentration was used for the production of naphthoquinone pigments. The major pigment present in *A. euchroma* is a shikonin derivative, i.e. acetylshikonin, as confirmed from HPLC analysis (**Fig. 5A-C**). High acetylshikonin yield was recorded in APM having alkaline pH (8.00 and above) (**Table 3**). It seems that higher pH (>8.00) of APM acts as a stressing agent, thereby triggering the activity of enzymes involved in secondary metabolism. The production of shikonin derivatives involves intermediates from two different pathways (mevalonate and phenylalanine pathway) and geranyl diphosphate: 4-hydroxybenzoate 3-geranyltransferase is reported to be a key regulatory enzyme, which combines the precursors from these two pathways to produce shikonin derivatives (Yazaki *et al.* 2002). This enzyme has optimum activity at pH 7.1-9.3 (Heide and Tabata 1987). Hence, our findings of pigment production at alkaline pH of APM could be correlated with enhanced activity of geranyl di-phosphate:4-hydroxybenzoate 3-geranyltransferase, thus regulating the production of naphthoquinone pigments. No doubt that the elevated pH resulted in high pigment production but it also hampered cell growth. The light microscopic studies revealed that the cells growing at alkaline pH were plasmolysed (**Fig. 6**), which might have resulted from the collapse of transmembrane potential. Lipophilic weak acids are known to collapse the membrane potential by shuttling protons across the membrane, thus resulting in release of metabolite granules into the intracellular spaces or medium (Mitchell 1966).

CONCLUSIONS

Non-growth linked pigment production behaviour in cell suspension culture of *Arnebia euchroma* clearly suggests the development of a two-stage culture protocol. Cell growth and production of naphthoquinone pigments was observed to be greatly influenced by pH of the respective media. Acidic pH (5.75-6.50) favours cell growth, whereas alkaline pH (8.00) is appropriate for pigment production; however, at pH extremes, cell growth seems to be hampered. Thus, it can be concluded that pH plays a significant role in cell metabolism affecting the growth as well as metabolite production.

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