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Enhanced production of plumbagin in immobilized cells of *Plumbago rosea* by elicitation and in situ adsorption

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Abstract

Cell cultures of *Plumbago rosea* were immobilized in calcium alginate and cultured in Murashige and Skoog's basal medium containing 10 mM $CaCl_2$ for the production of plumbagin, an important medicinal compound. Studies were carried to find out the impact of immobilization on the increased accumulation of this secondary metabolite. Immobilization in calcium alginate enhanced the production of plumbagin by three, two and one folds compared to that of control, un-crosslinked alginate and $CaCl_2$ treated cells respectively. Cell loading at a level of 20% to the polymer volume (Na-alginate) was optimal and maximum plumbagin was obtained. At higher cell loading (40–50%), lower plumbagin accumulation was noticed. Addition of 200 mg l^{-1} chitosan as an elicitor to the immobilized cells resulted in eight and two folds higher accumulation of plumbagin over control and immobilized cells. Also, more than 70% of the plumbagin was released into the medium, which is highly desirable for easy recovery of the product. Sucrose utilization rate of the cells was higher when cells were subjected to in situ product removal using Amberlite XAD-7. This may indicate that the toxicity of plumbagin was reduced on cells when it was removed from the medium. Cells subjected to combined treatments of chitosan, immobilization and in situ extraction showed a synergistic effect and yielded 92.13 mg g^{-1} DCW of plumbagin which is 21, 5.7, 2.5 times higher than control, immobilized, immobilized and elicited cells respectively.

Keywords: *Plumbago rosea*; Cell cultures; Plumbagin; Immobilization; In situ recovery

1. Introduction

Plumbagin (5-hydroxy, 2-methyl, 1-4 naphthoquinone) is an important naphthoquinone which shows a broad range of pharmaceutical activities such as anticancer (Krishnaswamy and Purushothaman, 1980; Parimala and Sachdanandam, 1993), leishmanicidal (Kayser et al., 2000), anti-

Abbreviations: IAA, indole-3-acetic acid; NAA, naphthaleneacetic acid; BAP, 6-benzylaminopurine; DCW, dry cell weight.

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microbial (Didry et al., 1994), antifertile (Bhargava, 1984), antimutagenic and insecticidal (Kubo et al., 1983). In vitro production of plumbagin by plant cell cultures is receiving more attention because native plants such as *Plumbago spp*., *Drosophyllum spp*., produce only small amounts of this compound after 2–6-years of growth (Kitanov and Pashankov, 1994). Production of plumbagin from *P. rosea* cell cultures have been reported earlier (Komaraiah et al., 2001). But, these cultures produced only 4.31 mg g^{-1} DCW of plumbagin and this amount is very small for commercial exploitation. Though plant cell cultures could be a potential source of a wide variety of valuable pharmaceuticals, only a few commercial processes based on plant cell cultures exist at the moment. The main drawback of cultured plant cells is lower yields, stability of the cell lines, inconsistency in the production and the storage of the metabolites within the cells or vacuoles. Recovery of products from cultures needs harvesting and extraction of the cell suspensions. The time and expenses in regenerating biomass and down stream processing ultimately make the process uneconomical. Many strategies were applied to the plant cell cultures such as elicitation, immobilization, permeabilization and continuous product recovery from the medium using two-phase systems for the product enhancement and recovery (Asada and Shuler, 1989; Curtis et al., 1995; Shim et al., 1999). Immobilized cell systems have advantages over free cells because of increased degree of differentiation/or cell to cell contact, which is very essential for the synthesis of secondary metabolites (Madhusudhan and Ravishankar, 1996; Komaraiah et al., 2001). But, many secondary metabolites are rarely released from the suspended or immobilized cell cultures, and they have very low solubility in water due to their hydrophobicity. In order to use immobilized cells more economically, it is necessary that the water insoluble products should be removed from the culture medium without disturbing the cell metabolic activity. In this connection, in situ adsorption or extraction of metabolites by using hydrophobic materials received great importance. Berlin et al. (1984) first reported the use of adsorbents to retain volatile compounds from cell cultures of *Thuja*. Kim and Chang (1990) obtained the enhanced shikonin by in situ extraction from calcium alginate immobilized cells of *Lithospermum*. In many cases in situ product removal has enhanced secondary metabolite production, and the products were selectively released from the cells and dissolved in the solvents or adsorbents (Fu and Lu, 1999; Shim et al., 1999; Wang et al., 2001).

The main intent of this study is to demonstrate the combined effects of an immobilized cell system and an artificial accumulation site for continuous removal of metabolites without harvesting the cells. In this investigation, we report the effects of immobilization on *Plumbago rosea* cell cultures, elicitation, and in situ removal of plumbagin using Amberlite XAD-7 and their synergistic effects on plumbagin synthesis and release.

2. Materials and methods

2.1. Cell cultures

Leaf derived cell cultures of *Plumbago rosea* L. (Komaraiah et al., 2001) were maintained in 250 ml Erlenmeyer flasks containing 60 ml of Murashige and Skoog's (MS) basal medium (1962) supplemented with 1 mg l^{-1} IAA, 0.5 mg l^{-1} NAA, 0.3 mg l^{-1} BAP and 3% sucrose. Cultures were grown on a gyratory shaker at 100 rpm with a 12-h photoperiod (30 μE m^{-2} S^{-1}) at 25±2 °C. The pH of the media was adjusted to 5.7 before autoclaving. Suspension cultures of *P. rosea* were harvested on the 16th day after subculture and resuspended into 2.5% alginate at cell loading density of 10–50% (fresh wt./polymer volume). The cell-alginate mixture was extruded drop wise in 0.1 M $CaCl_2$ using a syringe. The beads were kept in the solution for 30 min to ensure that the precipitation reaction reached completion and were rinsed with distilled water three times to remove traces of $CaCl_2$. The immobilized and free cells (2 g FW/100 ml) were cultured in MS medium (10 mM $CaCl_2$ was supplemented for immobilization studies) with or without an elicitor and Amberlite XAD-7. Chitosan was added into the medium on the 6th day of culture. Cultures were incubated on an orbital shaker at 120 rpm under

12-h light condition at 25±2 °C. Periodically, cultures were harvested, the cells and the media were analyzed for plumbagin production.

2.2. Chitosan and extraction of plumbagin from beads

One g of crab shell chitosan was dissolved in 2 ml of glacial acetic acid by adding drop wise at 60 °C for a period of 15 min and the final volume was made up to 100 ml. The pH of the solution was adjusted to 5.7 with NaOH before autoclaving and used as an elicitor. To determine the concentration of plumbagin in immobilized cells, calcium alginate beads were dissolved in 0.2 M citrate buffer (pH 5.0) and shaken for 30 min at 30 °C. The cells were rinsed with water and plumbagin was extracted.

2.3. Determination of plumbagin and HPLC analysis

After each experiment, the medium and cells were separated by filtration and the spent medium was extracted three times with equal volumes of ethyl acetate. The pooled fractions were reduced under vacuum and the residues redissolved in 1 ml of HPLC mobile phase. The cells were extracted thrice with methanol in a porcelain mortar using acid washed neutralized sand. Methanol was reduced under vacuum and redissolved in 1 ml of HPLC mobile phase and quantified. Data shown are mean of six replicates and ±S.E. values are presented as error bars. Plumbagin content in independent samples was determined by HPLC using two Shimadzu LC 10 AD pumps; SPD 10 A UV-VIS detector, and the column used was μ Bondapak C_{18} (3.9×300 mm^2) with a detection wave length of 254 nm. The mobile phase was methanol, water (80:20) with 0.1% trifluoroacetic acid and the peak area was calculated by comparing with an authentic sample of plumbagin obtained from the Sigma Chemical Company, USA.

2.4. Pretreatment of Amberlite XAD-7 and extraction of plumbagin from XAD-7

Prior to use, the resin was soaked in methanol for 24 h and then washed with several volumes of distilled water in a Buchner funnel. The washed resin was air dried in the same funnel by applying vacuum on the filter. One g of dried resin was weighed into 30 μm mesh nylon bag and tied properly. These bags were autoclaved in culture medium for 15 min to minimize the reduction of undeserved adsorption of medium compounds. The culture flasks were supplied with a nylon mesh bag with 1 g XAD-7 and were replaced with a new bag after every 3 days. Plumbagin concentration in cells/beads, medium and resin was determined by HPLC. Plumbagin content adsorbed onto the XAD-7 was determined by adding 100 ml of methanol to each nylon bag and extracted for 24 h on a shaker. Methanol was evaporated under vacuum and the residue was redissolved in 1 ml of HPLC mobile phase and analyzed on HPLC.

2.5. Analytical methods

Viability of the cells was tested by flouraceine diacetate technique (Widholm, 1972). Sucrose concentration in the medium was determined by the dinitrosalicylic acid method and by measuring the absorbency at 550 nm.

3. Results and discussion

3.1. Effect of cell immobilization in calcium alginate

P. rosea cells were immobilized in calcium alginate and cultured in MS medium containing 10 mM $CaCl_2$ to give strength to the beads. To study the impact of immobilization on *P. rosea* cells, the non-gelled alginate and $CaCl_2$ on cell growth and plumbagin production were examined (Figs. 1 and 2). Cells grown in 10 mM $CaCl_2$ exhibited normal growth, but the biomass was not as high as that of control cells. It has been reported that uncross linked alginate can act as carbohy-

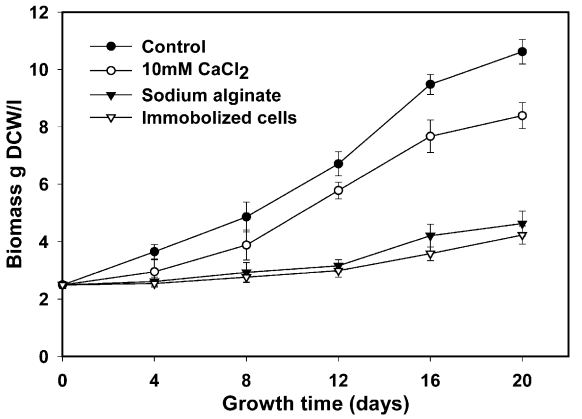

Fig. 1. Effect of immobilization and components on growth of *P. rosea* cell cultures.

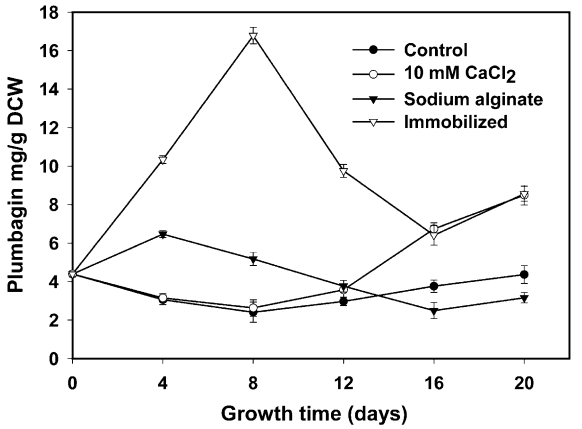

Fig. 2. Effect of immobilization and components on plumbagin accumulation in *P. rosea* cell cultures.

drate elicitor of secondary metabolism (Johnson et al., 1991). Though in the present study uncross linked alginate stimulated plumbagin synthesis almost two folds than the control cells (Table 1), cell growth was inhibited and over 40% of the cells lost their viability (data not shown). This may be due to the effect of alginate, which modifies the medium viscosity leading to poor oxygen transfer (Curtis et al., 1995). Immobilization of *P. rosea* cells in calcium alginate substantially enhanced the production of plumbagin. Maximum amount of plumbagin was recorded on the 8th day of culture but the concentration decreased thereafter rapidly. This could be due to degeneration of the secondary metabolite when it is present in higher concentrations (Wu et al., 1999; Yuan et al., 2001). Immobilized cells produced three, two and one-folds higher plumbagin to that of the control, uncross linked alginate and 10 mM $CaCl_2$ treated cells respectively (Fig. 2). This could be due to the effect of immobilization which can provide better cell to cell contact and also help in cell differentiation to produce secondary metabolites (Brodelius, 1983; Curtis et al., 1995). A similar pattern was observed in *Datura innoxia* cell cultures (Gontier et al., 1994). Besides, immobilization also influenced the extracellular product formation and more than 40% of the product was released into the culture medium (Table 1). This shows that the immobilization can effectively influence the physiology of cells (Brodelius, 1983; Curtis et al., 1995).

3.2. Cell concentration in calcium alginate beads

In order to optimize cell loading or concentration in alginate beads experiments were carried out using various cell densities to the polymer concentration (Fig. 3). Cell loading at 20% level was optimal and maximum plumbagin was noticed, but in the case of higher concentrations (40–50%), lower metabolite accumulation was recorded. At this stage, cells started coming out from the beads and the bead strength was also affected. The lower metabolite accumulation at higher concentration of cell loading may be due to the limitation of nutrients and the availability of the dissolved oxygen to the cells.

3.3. Elicitation of immobilized cells

Optimum concentration of chitosan for immobilized cells was tested (data not shown). In the case of free cells it was effective at 150 mg l^{-1} (Komaraiah et al., 2002), but in immobilized cells the effective dose of chitosan was 200 mg l^{-1} and this may be due to the diffusion barrier of immobilized beads to reach the cells. Addition of 200 mg l^{-1} chitosan to the immobilized cells produced eight and two folds higher plumbagin over control and immobilized cells when considered the most suitable time for plumbagin produc-

Table 1
Effect of different treatments on production pattern of plumbagin in *P. rosea* cell cultures

Type of treatment	Plumbagin content (mg g^{-1} DCW)				
	Intracellular	Extracellular	Total	% Release	Day of harvest
Control	3.52	0.85	4.37	19.45	16
Elicitation	7.65	21.29	28.94	73.56	18
Immobilization	8.41	7.73	16.14	47.89	8
10 mM $CaCl_2$	6.38	2.03	8.41	24.13	18
Uncross linked alginate	4.68	1.81	6.49	27.88	4
Immobilization+elicitation	9.64	26.53	36.17	73.34	10
Immobilization+elicitation+in situ product removal	15.63	76.50*	92.13	83.03	16

Control: Cultures were grown in standard growth medium (SGM) containing MS salts with 3% sucrose, 1 mg l^{-1} IAA, 0.5 mg l^{-1} NAA, 0.3 mg l^{-1} BAP; *Elicitation*: Free cells cultured in SGM were added 150 mg l^{-1} of chitosan and harvested after 48 h; *Immobilization*: Immobilized cells cultured SGM containing 10 mM $CaCl_2$; 10 mM $CaCl_2$: Cells cultured in SGM supplemented with 10 mM $CaCl_2$; *Uncross linked alginate*: Cells cultured in SGM supplemented with 2.5% sodium alginate; *Immobilization+chitosan*: Immobilized cells cultured in SGM medium containing 10 mM $CaCl_2$ and 200 mg l^{-1} of chitosan was added on the 8th day; *Immobilization+elicitation+in situ product removal*: Immobilized cells cultured in SGM containing 10 mM $CaCl_2$ with 1 g of XAD-7 in a nylon bag, chitosan was added on the 8th day of culture and incubated for another 8 days. All the cultures were maintained on a gyratory shaker at 100 rpm with 12 h photoperiod at 25 °C.

* The cells subjected to in situ product adsorption, most of the extracellular product was adsorbed onto the XAD-7.

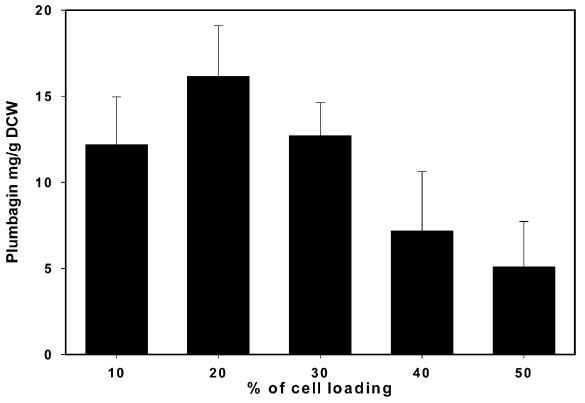

Fig. 3. Effect of cell concentration in immobilized beads on plumbagin production in *P. rosea* cell cultures.

tion in each case. As a natural, biocompatible, cationic biopolymer, chitosan is safe to use and advantageous because of its effect on cell permeabilization (Johnson et al., 1991), in the present study more than 70% of the plumbagin was released into the medium (Table 1). This is highly desirable for the extraction of the metabolite more economically.

3.4. Sucrose utilization

The effect of sucrose utilization rate of *P. rosea* cell cultures is presented in Fig. 4. The cells subjected to in situ extraction consumed more sucrose than the cells that have not been added XAD-7. This shows that XAD-7 did not show any adverse effects on cell metabolism. In fact, the cells utilized sucrose more rapidly and this may indicate the reduced toxicity of secondary metabolite on

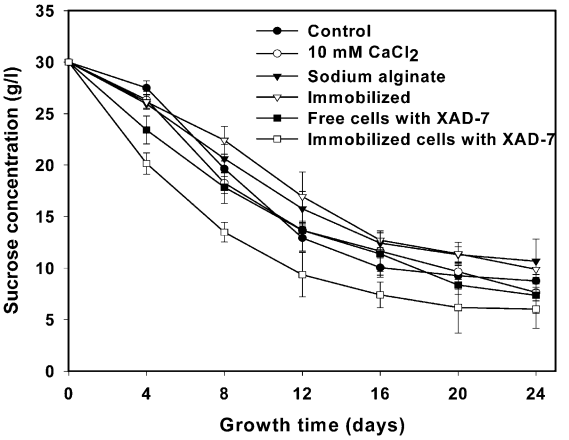

Fig. 4. Effect of different treatments on sucrose consumption of *P. rosea* cell cultures.

cells due to its adsorption by XAD-7 as pointed out by Pavao et al. (1996) and Kown et al. (1998).

3.5. *In situ* plumbagin adsorption

It is important to remove the water insoluble compounds/metabolites from the medium, which are not only toxic to the cells at higher concentrations but also their presence for longer duration may eventually lead to product degeneration. This demonstrates that in situ extraction of metabolites with organic solvents or adsorption onto non-ionic polycarbonated beads was more successful (Kown et al., 1998; Fu and Lu, 1999). Synergistic effect of immobilization, elicitation and in situ product adsorption yielded a maximum amount of 92.13 mg of plumbagin g^{-1} DCW. The total metabolite production was 21, 5.7, 3.1 and 2.5-times higher than free cells, immobilized cells, free cells with chitosan elicitation and immobilized cells elicited with chitosan respectively. These results suggest that in situ removal of plumbagin using XAD-7 as a second phase reduced feed back inhibition of secondary metabolite synthesis. A similar pattern of results were reported in *Rubia akane* and *Taxus* suspension cultures when they were subjected to in situ product recovery (Kown et al., 1998; Shim et al., 1999).

4. Conclusions

Elicitation of immobilized *P. rosea* cells with chitosan proved to be highly effective by using an extracellular site for the product accumulation. The production of plumbagin increased about 21-times by collective use of immobilization, elicitation and two-phase culture. Most of the plumbagin released into the medium was adsorbed onto XAD-7, which is highly desirable to extract the product more economically and to get rid of the toxic effects on the cells. Since no single treatment resulted in significant accumulation of product, integrated process design could be a very good strategy to improve the yields of plant cell cultures.

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