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INCREASED SHIKONIN PRODUCTION BY HAIRY ROOTS OF *LITHOSPERMUM ERYTHRORHIZON* IN TWO PHASE BUBBLE COLUMN REACTOR

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SUMMARY

Plant hairy root cultures of *Lithospermum erythrorhizon* were carried out to produce shikonin derivatives by employing *in situ* extraction with *n*-hexadecane in a shake flask and a bubble column bioreactor. Over 95 % shikonin produced was recovered in the *n*-hexadecane layer. In flask cultures the maximum concentration of shikonin with *n*-hexadecane extraction was 3 times higher than that obtained without extraction. In the two phase bubble column reactor, 572.6 mg/L of shikonin and 15.6 g/L of dry cell mass were obtained after 54 days. Shikonin was produced at a constant level of 10.6 mg/L·day during this period.

INTRODUCTION

Recently, plant hairy roots have been drawing increased interest because of their genetic and biochemical stability, fast growth rate, and their ability to synthesize useful products comparable to the original plant roots (Hamill *et al.*, 1987). However, most secondary metabolites obtained from hairy root cultures are typically located within cell vacuoles or cell walls, which results in inhibition of cell growth and further synthesis of products. Also, hairy roots had to be harvested and disrupted to recover products. It is, therefore, desirable for the products to be released from hairy roots into the culture medium so that continuous operation can be carried out. There have been several reports including the recent one by Green *et al.* (1992) that showed the intracellular and extracellular levels of secondary metabolites could be altered using different culture conditions. *In situ* adsorption and extraction have been widely applied for the removal of inhibitory products in the field of biotechnology including the plant cell cultures. Brodelius and Nilsson (1983) showed that some solvents were useful to extract products from immobilized plant cells without affecting cell viability.

Shikonin is the most successful plant secondary metabolite produced in large scale by cell suspension culture (Fujita and Tabata, 1987). Shikonin production by cell suspension culture

in two-phase systems was reported by Deno *et al.* (1987). Kim and Chang (1990) were able to enhance shikonin production by employing *in situ* extraction and cell immobilization. Shimomura *et al.* (1991) also applied this culture method to the hairy roots.

In this paper the results obtained from immobilized hairy root culture of *L. erythrorhizon* in a two phase bubble column reactor are reported. The effects of *in situ* extraction using *n*-hexadecane in flasks and a bubble column reactor are also discussed.

MATERIALS AND METHODS

Hairy root cultures

A hairy root of *Lithospermum erythrorhizon* transformed by *Agrobacterium rhizogenes* A4 (Seo et al., 1992) was used in this study. Hairy roots were subcultured in three-fold diluted SH medium (Schenk and Hildebrandt, 1972) containing 30 g/L sucrose and no hormone. The culture medium used in the experiments was modified SH medium containing no phytohormone and salts at the following concentrations: 2.5 g/L potassium nitrate, 100 mg/L ammonium phosphate, and 0.125 mg/L cupric sulfate. The medium was supplemented with 40 g/L sucrose and pH was adjusted to 5.8 with 0.5 N NaOH/HCl. Medium was sterilized by autoclaving for 15 min at 121 °C and 15 psig. A 250 ml Erlenmeyer flask containing 50 ml medium was inoculated with a lateral branch (approximately 100 mg fresh weight) and grown under dim light conditions at 25 °C in a rotary shaker at 100 rpm. Sterile *n*-hexadecane was also added to the inoculated flasks when needed for *in situ* extraction.

Bioreactor system

The bubble column bioreactor of inner diameter of 80 mm and the height of 400 mm (working volume of 1.5 L) was constructed. A stainless steel mesh was installed inside the reactor to immobilize hairy roots. Humidified air was introduced into the column through a sintered glass sparger at the bottom. Hairy roots were aseptically anchored to regular positions on the stainless steel mesh. To prevent the mixing of medium and solvent during the exchange, different inlet and outlet ports were used. The culture broth in the reactor was aerated by supplying filtered air at a rate of 0.05-0.2 vvm through the sintered glass sparger. Temperature was maintained at 25 °C by circulating constant temperature water through the jacket.

Analytical methods

Sugar concentrations were determined by HPLC (L3300 RI detector, Hitachi, Ltd., Japan) equipped with an ion-exclusion column (Bio-Rad HPX-87H, CA, USA) and a refractive index detector using 0.01 N sulfuric acid as eluent (flow rate of 0.4 mL/min). Shikonin derivatives were extracted from fresh cells with chloroform. The amounts of shikonin derivatives in cell mass and *n*-hexadecane layer were determined by the methods described by Deno *et al.* (1987). The concentration of phenolics secreted from hairy roots into the culture medium was determined by the procedure adapted from Singleton and Rossi (1965). For cell mass determination in flasks, the hairy roots were harvested by paper filtration, rinsed with large volume of distilled water, and dried at 60 °C for 24 h before gravimetrical weight determination. During bioreactor operation cell growth was indirectly estimated by conductivity measurement (Taya *et al.*, 1989). There was a linear relationship between cell mass and conductivity, which could be shown as

$$\Delta\kappa = K(\Delta X)$$

where variables were defined as X for dry cell mass concentration, K for an empirical coefficient, and κ for specific conductivity. In this experiment K was found to be 3.89 mS/g·cm^2.

RESULTS AND DISCUSSION

Shake flask culture

Shake flask cultures were first carried out to investigate the time profile of shikonin production and sugar consumption. Dry cell mass and shikonin production in the shake flask cultures are shown in Fig. 1(a). Shikonin was detected from day 6 and increased continuously until day 21. Dry cell weight of hairy root increased to 3.3 g/L after 17 day and did not increase further most likely due to the accumulation of shikonin and other inhibitory products. Total shikonin concentration started to decrease from day 21. Product degradation may be responsible for the decrease of total shikonin concentration. Shikonin concentration in hairy roots decreased even faster. However, shikonin concentration in the culture medium continuously increased. This was due to the release of shikonin from broken and detached hairy roots as will be discussed in the next section.

Carbohydrate consumption and formation of total phenolics are shown in Fig. 1 (b). Sucrose concentration decreased almost linearly until the cell growth stopped, after which sucrose was hydrolyzed more rapidly. This was accompanied by sharp increase of the concentration of glucose and fructose. Hydrolyzed glucose was preferentially utilized over fructose by hairy roots as observed previously in the cell suspension cultures (Nikolova et al., 1991). To investigate the possibility of product formation other than shikonin, the concentration of phenolics secreted from hairy roots was measured. As expected, amount of phenolics increased in spite of cessation of cell growth and shikonin synthesis. Therefore, carbohydrates taken up by cells were utilized to synthesize other secondary metabolites different from shikonin.

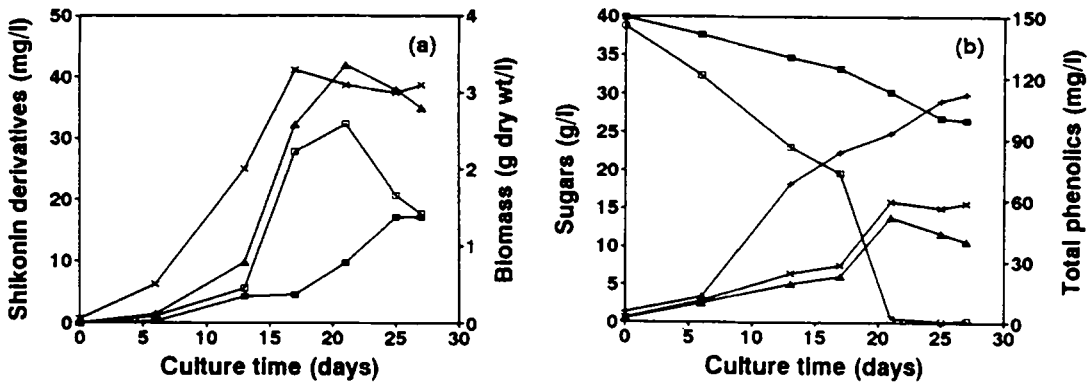

Figure 1. Time courses of shikonin production and sucrose consumption in shake flask culture. (a) Δ: total shikonin; □: shikonin in hairy root; □: shikonin in medium; X: dry cell weight. (b) □: total sugar; □: sucrose; X: fructose; Δ: glucose; +: total phenolics.

To understand the detailed mechanism of the cessation of shikonin production and cell growth, the effects of nutrient deletion need to be investigated.

Effect of *in situ* extraction on shikonin production in flask culture

To investigate the effects of *in situ* product removal from hairy root flask culture, various amounts of *n*-hexadecane (10, 20, 30 and 40 mL) were added to 50 mL modified SH medium. Hairy roots were cultivated for one month (Fig. 2). Most of the shikonin produced (more than 95 %) was recovered in the *n*-hexadecane layer. Shikonin concentration decreased after 20 days in the flask culture without extraction. For the cultures with *n*-hexadecane extraction, shikonin concentration did not decrease during the whole period. Using 30 ml *n*-hexadecane gave the best results and final shikonin concentration was 3 times higher than that obtained without extraction (Table I). As the volume of *n*-hexadecane increased up to 30 ml, shikonin production was enhanced by efficiently removing shikonin produced and other products which might be inhibitory to cell growth. However, using a volume higher than 30 ml of *n*-hexadecane resulted in less shikonin production probably due to oxygen limitation in the culture medium as mentioned by Kim and Chang (1990) and Byun *et al.* (1990). It is, however, expected that shikonin production will be enhanced in this system when oxygen can be supplied efficiently as shown in next section.

It is also important to see that shikonin could be detected earlier using higher volume of *n*-hexadecane.

Another thing that should be mentioned is the effect of *n*-hexadecane presence on the growth and the shape of hairy roots. As can be seen from Table 1, final dry cell weight is higher for the

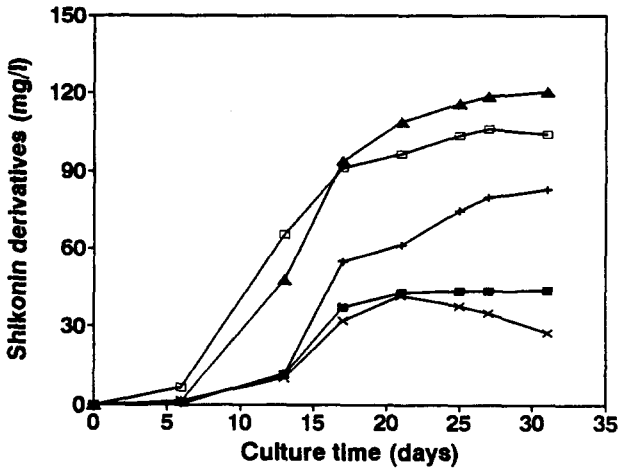

Figure 2. Time courses of shikonin production by hairy root cultures with *in situ* extraction with *n*-hexadecane. □: 10 ml; +: 20 ml; Δ: 30 ml; ■:40 ml *n*-hexadecane added to 50 ml modified SH medium, X: without *in situ* extraction.

culture with larger volume of solvent. This is due to the removal of shikonin and other metabolites that would be growth inhibitory if present. The shapes of hairy roots were observed under microscope. When hairy roots were grown without *n*-hexadecane, damaged root hairs were visible, some of which were physically detached from main body. Hairy roots grown in the presence of solvent looked healthy and longer. Therefore, it can be said that presence of *n*-hexadecane indirectly supports better growth of healthy roots by *in situ* extraction of shikonin, which becomes growth inhibitory when present at higher concentration.

Table I. Effect of *in situ* extraction by *n*-hexadecane on shikonin production (inoculum concentration = 0.1 g/L-medium).

Volume of added solvent (mL)	Culture time[a] (day)	Maximum shikonin conc. (mg/L)	Final dry cell weight (g/L)	Cellular productivity[b] (mg/g·h)	Volumetric productivity (mg/L·day)
0	20	42.0	3.2	0.88	2.1
10	21	43.8	3.3	0.87	2.1
20	26	82.8	3.8	1.33	3.2
30	26	120.6	5.0	1.94	4.7
40	26	106.2	5.0	1.71	4.1

[a] Time for maximum shikonin concentration
[b] Based on inoculated dry cell mass

Two phase bubble column reactor operation

Two phase bubble column reactor was constructed and used to possibly improve shikonin production. Approximately 100 mg of hairy root cells grown for 10 days in three fold diluted SH medium were transferred to the reactor containing modified SH medium and *n*-hexadecane at pH 5.8. The volumes of culture medium and *n*-hexadecane were 600 and 300 mL, respectively. Higher volume ratio of solvent to medium than that used in flask culture was due to sampling.

The results are shown in Fig. 3. Medium and solvent were exchanged when the sucrose consumption decreased as shown by down arrows. The hairy roots grew much better and the reactor was almost packed with hairy roots (data not shown).

Shikonin production increased continuously during 54 days of the experiments. The final cell mass and total shikonin produced were 15.6 g DCW/L and 572.6 mg/L, respectively. Shikonin was produced at a constant level of 10.6 mg/L·day during a culture period of 54 days.

There was one problem that needs to be resolved for long term operation in this reactor configuration. Channeling occurred in the reactor as hairy roots grew and aggregated, which might have caused oxygen limitation in a certain region. For long term operation this should be avoided.

From the above results, it can be concluded that hairy root culture with *in situ* extraction is useful for shikonin production and effective recovery. Shikonin production can be further improved by using the two phase bubble column reactor as shown in this study. This technique may be useful for production of other useful secondary metabolites from plant hairy root culture and may also allow continuous operation.

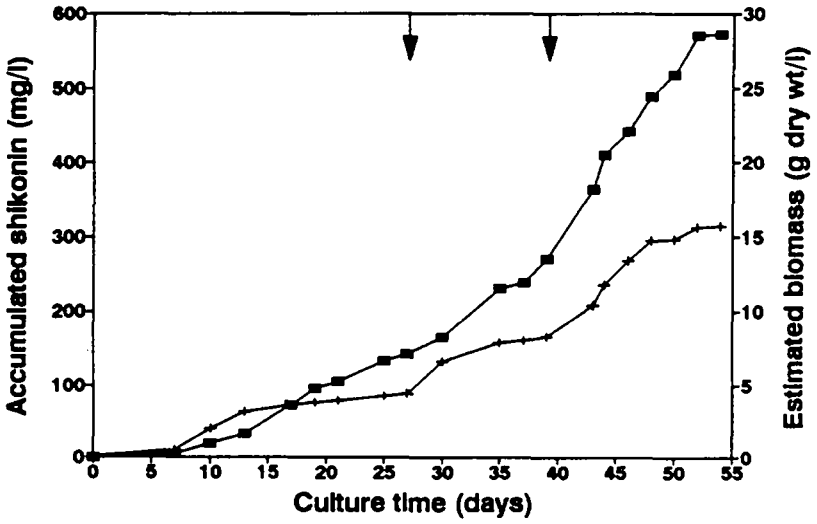

Figure 3. Time courses of hairy root growth and shikonin production in two phase bubble column bioreactor. +: dry cell weight; □: total shikonin; arrow: exchange of medium and solvent.

Acknowledgements

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