Plant Cell Reports (1981) 1: 59–60

Plant Cell Reports

Production of Shikonin Derivatives by Cell Suspension Cultures of *Lithospermum erythrorhizon*

I. Effects of Nitrogen Sources on the Production of Shikonin Derivatives

Yasuhiro Fujita, Yasuhiro Hara, Takao Ogino, and Chuzo Suga

Research Center, Mitsui Petrochemical Industries, Ltd., 6-1-2, Waki-cho, Kuga-gun, Yamaguchi-ken, 740, Japan

Received August 14, 1981

ABSTRACT

Shikonin derivatives were produced from cell suspension cultures of *Lithospermum erythrorhizon* for the first time. The results of studies on various culture media showed that the type of nitrogen source in the medium greatly influenced the production of these derivatives. When nitrate was the nitrogen source, stable production of shikonin derivatives by the cell suspension culture took place. We also found that ammonium as the nitrogen source conspicuously inhibited the production of shikonin derivatives.

INTRODUCTION

Shikonin derivatives from the roots of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) possess antibacterial activity and stimulate the formation of granulation tissue, thus, these derivatives have been used as topical medications for wounds, burns, hemorrhoids, etc.

Tabata et al. (1974) found that callus tissue of *L. erythrorhizon* produced shikonin derivatives on Linsmaier-Skoog agar medium. Mizukami et al. (1977, 1978) investigated the conditions under which these derivatives are produced by callus cultures.

We tried to produce shikonin derivatives from cell suspension cultures grown in the Linsmaier-Skoog medium. Unexpectedly, none were produced, although the cells grew well. Therefore, we investigated the effect of the composition of the media on shikonin production. Cell suspensions of *L. erythrorhizon* were cultured in five widely used media; White's (1954), Linsmaier-Skoog (1965), Blaydes' (1966), Gamborg et al.'s B-5 (1968), and Nitsch and Nitsch's (1969) medium. We here discuss the relation between productivity and the medium.

MATERIALS AND METHODS

Cultural Methods. A *L. erythrorhizon* cell line, M-18 (Mizukami et al., 1978), obtained from Prof. M. Tabata, Faculty of Pharmaceutical Science, Kyoto University, was used. This cell line was subcultured on Linsmaier-Skoog agar medium supplemented with 10^{-6} M 3-indoleacetic acid and 10^{-5} M kinetin. The cells then were transferred to Linsmaier-Skoog liquid medium and subcultured every 14 days. Cells harvested 8-10 days after subculture, which were in the late logarithmic growth-phase, were used for an experiment on the production of shikonin derivatives.

These cells were separated from the medium by filtering them through a stainless steel sieve with a pore size of 40 μ, after which they were inoculated into the various media. The effect of a given medium on shikonin productivity was investigated. All the media were supplemented with 10^{-6} M 3-indoleacetic acid and 10^{-5} M kinetin. The inoculum consisted of 0.5 g fresh wt. of tissue per 27 ml medium in a 100 ml flask.

All cultures were carried out at 25°C in the dark. The cell suspensions were agitated on a rotary shaker at a rotation speed of 100 rpm with an agitation diameter of 25 mm.

Determination of Shikonin Derivatives. When the culture period of 14 days had ended, the cells were harvested, and dried at 35°C for 24 h. Shikonin derivatives were extracted by ethanol and were determined by the methods described by Mizukami et al. (1977).

RESULTS AND DISCUSSION

Cell line M-18 could produce shikonin derivatives on the Linsmaier-Skoog agar medium, but not in their liquid medium, although these cells grew well even in the liquid medium. We compared cell-growth and the yield of shikonin derivatives by culturing cell samples in five media; White's(1954), Linsmaier-Skoog (1965), Blaydes' (1966), Gamborg et al.'s B-5 (1968), and Nitsch and Nitsch's (1969) medium. The Linsmaier-Skoog medium was best for cell-growth and Gamborg et al.'s B-5 medium was next best (Table 1). The other media did not produce good cell-growth. Interestingly, of the five media, shikonin derivatives were produced only in White's medium. Thus, differences in the yield of shikonin derivatives as well as cell-growth depended on the type of medium used.

Table 1. Effects of the Type of Media on Cell-Growth and on the Production of Shikonin Derivatives.

Medium	Cell yield (g·DW/l)	Shikonin derivatives formed (mg/l)
White (1954)	6.8	130
Linsmaier & Skoog (1965)	16.8	0
Blaydes (1966)	4.0	0
Gamborg et al. B-5 (1968)	12.2	0
Nitsch & Nitsch (1969)	4.6	0

White's medium differed from the other media in respect to the nitrogen source. It contained only nitrate, whereas the other media contained nitrate and ammonium. The difference in productivity seemed to be due to the nitrogen source used.

We examined the relation between the nitrogen source

0721-7714/81/0001/0059/$ 01.00

and productivity with White's medium and the Linsmaier-Skoog medium by varying the contents of nitrate and ammonium. Regardless of the basal medium, no shikonin derivative was produced when the nitrogen source included ammonium (1.1 mM), but derivatives were produced when it consisted of nitrate only (Table 2). However, there was difference in productivity between the basal media. With 3.3 mM nitrate, the yield increased when White's medium was used. In contrast, cells grew better in the Linsmaier-Skoog medium than in White's medium.

Table 2. Influence of the Nitrogen Source on Cell-Growth and on the Production of Shikonin Derivatives.

NH_4^+ conc. (mM)	NO_3^- conc. (mM)	Basal medium	Cell yield (g·DW/l)	Shikonin derivatives formed (mg/l)
0	0	W [a]	1.8	0
1.1	2.2	W	2.1	0
1.1	2.2	LS [b]	2.8	0
0	3.3	W	3.8	100
0	3.3	LS	5.5	50

a White's medium without nitrogen
b Linsmaier-Skoog medium without nitrogen

As described above, the nitrogen source in the medium has a great influence on the production of shikonin derivatives; ammonium inhibits their production. To determine whether ammonium really did inhibit the production of shikonin derivatives, we changed the mole ratio of nitrate to ammonium under a constant concentration of the nitrogen source (NO_3^- +NH_4^+ =3.3 mM) in White's medium. As shown in Table 3, production was inhibited completely even with only 3% ammonium in the nitrogen source. Cell-growth was decreased at more than 10% ammonium.

Table 3. Relation of NH_4^+ Content to Cell-Growth and to the Production of Shikonin Derivatives.

NH_4^+ content (%)	Cell yield (g·DW/l)	Shikonin derivatives formed (mg/l)
0	5.6	130
1	5.7	60
3	5.5	0
5	5.4	0
10	5.6	0
30	2.6	0
50	2.1	0

Examples of the influence of various nitrogen sources on cell-growth is well documented (Riker and Gutsch, 1948; Nitsch and Nitsch, 1956), but little is known about their influence on the production of metabolites. One case of the influence of the type of nitrogen source on metabolite production has been reported (Ikeda et al., 1977) in cell suspension cultures of tobacco that produce ubiquinone. In this instance, an ammonium addition is described as the nitrogen source rather than nitrate only in order to increase the yield. But, for the production of shikonin derivatives, we found that the yield was higher when only nitrate was used than when nitrate and ammonium were used. We consider the inhibition by ammonium an interesting phenomenon and are making further studies of this phenomenon.

ACKNOWLEDGEMENTS

We thank Prof. Arasuke Nishi, Toyama Medical and Pharmaceutical Univ., Prof. Mamoru Tabata, Kyoto Univ., and Prof. Yasuyuki Yamada, Kyoto Univ. who gave us extensive advice and encouragement during these experiments.

REFERENCES

Blaydes DF (1966) Physiol. Plant. 18: 748-753
Gamborg OL, Miller RA, Ojima K (1968) Exp. Cell Res. 50: 151-158
Ikeda T, Matsumoto T, Noguchi M (1977) Agric. Biol. Chem. 41: 1197-1201
Linsmaier EF, Skoog F (1965) Physiol. Plant. 18: 100-127
Mizukami H, Konoshima M, Tabata M (1977) Phytochemistry 16: 1183-1186
Mizukami H, Konoshima M, Tabata M (1978) ibid. 17: 95-97
Nitsch JP, Nitsch C (1956) Amer. J. Bot. 43: 839-851
Nitsch JP, Nitsch C (1969) Science 163: 85-87
Riker AJ, Gutsch AE (1948) Amer. J. Bot. 35: 227-238
Tabata M, Mizukami H, Hiraoka N (1974) Phytochemistry 13: 927-937
White PR (1954) The Cultivation of Animal and Plant Cells, The Ronald Press Co., New York.