ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 11 (2000) 59–61

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Letter

High production of β-thujaplicin with *Thuja dolabrata* var. *hondai* cells in a semi-continuous culture system

Kazumi Sanada [a], Akihiko Kawaguchi [a], Tsutomu Furuya [b], Kohji Ishihara [c], Nobuyoshi Nakajima [d], Hiroki Hamada [b,*]

[a] *Graduate School of Arts and Science, Life Science, The University of Tokyo, Meguro-ku, Tokyo 153-8902, Japan*
[b] *Department of Applied Science, Okayama University of Science, Ridai-cho, Okayama 700-0005, Japan*
[c] *Department of Chemistry, Kyoto University of Education, Fushimi-ku, Kyoto 612-8522, Japan*
[d] *Department of Nutritional Science, Okayama Prefectural University, Soja, Okayama 719-1112, Japan*

Received 8 May 2000; received in revised form 8 June 2000; accepted 8 June 2000

Abstract

Cultured cells of *Thuja dolabrata* var. *hondai*, which were able to produce β-thujaplicin in high yield, were induced and selected. The effective production and extraction of β-thujaplicin has been achieved using the cultured cells in a semi-continuous culture system. This system needs only the whole cells without immobilizing them and the flesh medium in each batch production. The productive efficiency did not decrease during repetitive batch production.

Keywords: *Thuja dolabrata* var. *hondai*; Cell culture; β-thujaplicin; Hinokitiol; Semi-continuous culture system

β-Thujaplicin (hinokitiol), a naturally occurring tropolone derivative, has been widely used in Japan as a medicine, food ingredient and cosmetic due to its strong antibacterial activities. β-Thujaplicin is extracted from the heartwood sawdust of *Thuja dolabrata* var. *hondai* (Japanese name: hiba or aomori-hiba). Recently, the demand for β-thujaplicin is increasing, however, the supply of β-thujaplicin is quite limited due to regulated deforestation. To date, the production of β-thujaplicin by the cell suspension culture of *T. dolabrata* var. *hondai* [1], *T. occidentals* [2], and β-thujaplicin-containing related plants such as *Cupressus lusitanica* [3,4] and *Calocedrus formosana* [5], have been investigated, but satisfactory results have not yet been obtained. We now reported the high production of β-thujaplicin using a semi-continuous suspension cultures of *T. dolabrata* var. *hondai* cells.

T. dolabrata var. *hondai* leaves and seeds were obtained from Aomori Prefecture, Japan, in 1994. Calli were induced from the leaves and seedlings on Murashige and Skoog's (MS) medium [6] containing 3% sucrose, 1 ppm of 1-naphthalene acetic acid (N1), and 0.5% agar (adjusted to pH 5.8). The calli were subcultured at 4-week intervals on solid MS medium. A suspension culture was started by transferring the calli to 100 ml of liquid MS medium containing 3% sucrose and N1 (MS-N1, pH 5.8) in a 300 ml Erlenmeyer flask. The suspension culture was agitated on

* Corresponding author. Tel.: +81-86-256-9473; fax: +81-75-256-8468.
E-mail address: hamada@das.ous.ac.jp (H. Hamada).

1381-1177/00/$ - see front matter
PII: S1381-1177(00)00191-0

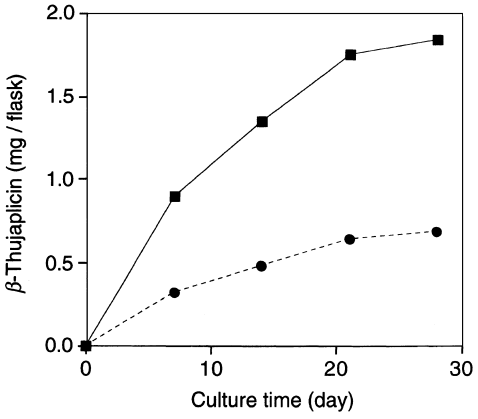

Fig. 1. Time course of β-thujaplicin production in T-3 cells. Amount of β-thujaplicin in medium (■) and cells (●).

a rotary shaker at 100 rpm and 25°C in the dark, and subcultured at 2 week intervals in the same freshly prepared medium. β-Thujaplicin-producing cells were easily distinguished from the non-producing ones by the color of the culture medium. Three days after the transfer into fresh medium, the medium became reddish-brown due to formation of the β-thujaplicin-Fe ion complex and an oil film on the surface was visible. No root and shoot like structures were found. The cells have been maintained for more than 5 years on MS-N1 medium. Intracellular β-thujaplicin was extracted from the fresh cells with 10 volumes of AcOEt after ultrasonic treatment. The used medium was extracted twice with AcOEt at the rate of 1–10 of the water volume. The extracts were concentrated under reduced pressure. The amounts of β-thujaplicin in the cells and the used medium were measured by HPLC (Crest Pak C18S Ø 4.6 mm × 250 mm, 0.1% H_3PO_4 in CH_3CN:H_2O = 55:45). The identification of the product was confirmed by ^{1}H- and ^{13}C-NMR and HPLC analyses on the comparison with a commercial available β-thujaplicin.

Twenty-six cell lines (T-1–T-26) were derived from calli and examined under the regulation of following plant growth hormones: N1 (1 ppm of 1-naphthalene acetic acid), N1K0.1 (N1 + 0.1 ppm of kinetin), D1 (1 ppm of 2,4-dichlorophenoxyacetic acid), D1K0.1 (D1 + 0.1 ppm of kinetin). The cell lines (each 1 g) were suspended into 100 ml of the four media (MS-N1, MS-N1K0.1, MS-D1, and MS-D1K0.1), and then the amounts of β-thujaplicin in the cell line and the cell growth were measured after incubation for 3 days. As the best cell line, the T-3 cell line regulated by N1K0.1 was selected (the amount of β-thujaplicin in the cell line: 236 μg, the cell weight: 1.2 g) and used in the following experiments.

The β-thujaplicin production from 1 to 4 weeks was studied in both T-3 cells and the medium. As shown in Fig. 1, it was observed that the production ratio of β-thujaplicin in cells and the used medium is about 1:3. After 3 weeks, the cell growth ratio was 2.36 times, and the β-thujaplicin content in the total oils from the cells and in the used medium were 23 and 92%, respectively.

The relationship of inocula size, e.g. 1, 3, and 5 g, into the fresh medium (100 ml) and the β-thujaplicin production in the medium was examined as shown in Fig. 2.

It was found that an inocula size of 1 g in the fresh medium (100 ml) produced the highest amounts of β-thujaplicin in the medium.

Repetitive use of the T-3 cell line in a semi-continuous culture system was investigated. A suspension culture was started by transferring 1 g of calli to 100 ml of the liquid MS-N1K0.1 medium in a 300 ml Erlenmeyer flask and cultured for 2–4 week. The used culture medium was changed to the same freshly prepared medium (sterilized) and continuously subcultured at 1 or 2 week intervals (see Fig. 3).

The weight of the cells increased about two times among the first batch production, however, the cell growth was not observed after the second batch

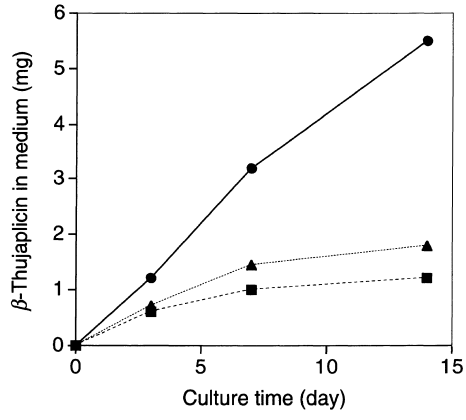

Fig. 2. Relationship between inocula size and β-thujaplicin production. Amount of β-thujaplicin in medium: inocula size, 1 g per 100 ml (●), 3 g per 100 ml (▲), 5 g per 100 ml (■).

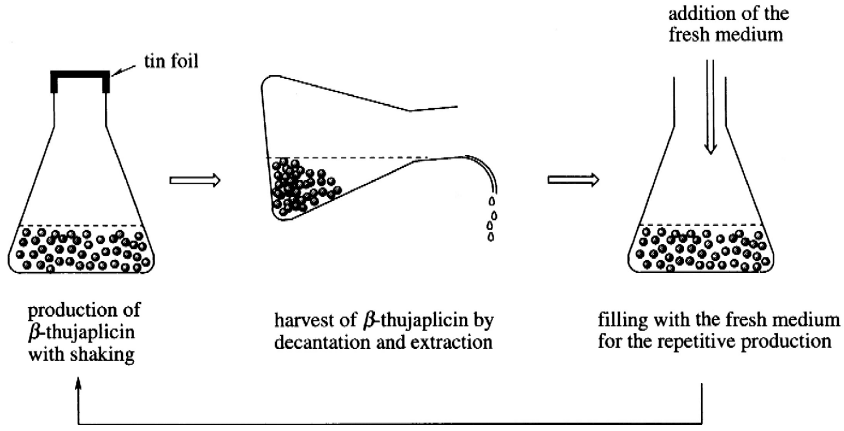

Fig. 3. Schematic diagram of a semi-continuous culture system.

production. The addition of fresh medium did not show the accumulation of intracellular β-thujaplicin, while the producing in extracellular β-thujaplicin in the medium was observed. In two cases, over 28 mg of β-thujaplicin was obtained after six batch productions as shown in Table 1.

In repetitive batch use, the T-3 cells maintained the high productivity after 12 weeks of usage. These results show that the semi-continuous culture system is better than the normal suspension culture (single use) for effective β-thujaplicin production. Furthermore, this system had an advantage of recovering β-thujaplicin produced, because the product was excreted into the used medium. Thus, the effective production of β-thujaplicin has been achieved using T-3 cells in a semi-continuous culture system. The application of this system to a large-scale (gram scale production) production of β-thujaplicin is currently under investigation and will be reported in a future paper.

Table 1
The production of β-thujaplicin by *T. dolabrata* var. *hondai* cells in a semi-continuous culture system[a]

Batch[b]	Run 1		Run 2	
	Time[c] (week)	Yield[d] (mg)	Time[c] (week)	Yield[d] (mg)
1	2	5.16	4	9.39
2	2	6.71	1	5.26
3	2	5.37	1	4.02
4	2	4.57	1	3.35
5	2	3.20	1	3.21
6	2	3.06	1	3.15
Total	12	28.07	9	28.38

[a] Initial conditions: 1 g of T-3 cell line was added to 100 ml of the fresh MS media containing 1 ppm of 1-naphthalene acetic acid and 0.1 ppm of kinetin.

[b] The media was exchanged with a freshly one in each batch reaction.

[c] Culture time.

[d] Amount of β-thujaplicin in used (cultured) medium.

Acknowledgements

We are grateful to Mr. F. Murakami and President Y. Shima, Seiwa Technological Laboratories, Ltd., for encouragement and the helpful discussions.

References

[1] R. Fujii, K. Ozaki, M. Ono, H. Watanabe, Plant Tissue Cult. Lett. 12 (1994) 55.
[2] R. Itose, K. Sakai, Plant Biotechnol. 14 (1997) 163.
[3] M. Ono, T. Asai, H. Watanabe, Biosci. Biotechnol. Biochem. 62 (1998) 1653.
[4] K. Sakai, K. Kusaba, Y. Tsutsumi, T. Shiraishi, Mokuzai Gakkaishi 40 (1994) 1.
[5] L. Witte, J. Berlin, V. Wray, W. Schubert, W. Kohl, G. Hoefle, J. Hammer, Planta Medica 49 (1983) 216.
[6] T. Murashige, F. Skoog, Physiol. Plant 15 (1962) 473.