J. Microbiol. Biotechnol. (1999), **9**(3), 358–360

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Improved Menthol Production Using Suspension Cultures of *Mentha piperita* with Pectinase Elicitation

CHANG, JUN HYONG, JOONG HAN SHIN, IN SIK CHUNG[1], AND HYONG JOO LEE*

Department of Food Science and Technology and Research Center for New-Biomaterials in Agriculture, Seoul National University, Suwon 441-744, Korea
[1]Department of Genetic Engineering, Kyung Hee University, Yongin 449-701, Korea

Received: December 7, 1998

Abstract The effect of pectinase on menthol production by *Mentha piperita* in shake flasks was investigated. The optimum concentration of pectinase and period of elicitation for menthol production were 15 U/l and 9 days, respectively. Pectinase elicitation at 15 U/l for 9 days using a Lin-Staba medium with 2,4-dichlorophenoxyacetic acid (2,4-D) enhanced menthol production 37-fold (211.5 mg menthol/l) with the specific menthol concentration (menthol concentration per unit weight of cells) of 27.5 mg/g dry cell weight (DCW). Our results also indicate that pectinase elicitation may activate the conversion of pulegone to menthol.

Key words: *Mentha piperita* L., elicitation, pectinase, menthol

Increased demand for natural food flavors has resulted in numerous studies on plant cell culture and the development of flavors such as mint oils, vanillin, limonene, geraniol, and quassin. Among these, peppermint oil is probably one of the most popular flavors [1, 9], and therefore, much research has been carried out on the callus or cell culture of peppermint [2, 7, 10]. Song and Lee [13] cultivated *M. piperita* cells in an air-bubble bioreactor, while Kim and Lee [8] tried to immobilize peppermint cells in polyurethane foams, and reported that 90% of peppermint cells were immobilized successfully.

A wide variety of elicitors have been employed to alter cell metabolism in order to enhance the production of secondary metabolites in plant cell cultures. Among these elicitors, the representative enzyme elicitors were cellulase and pectinase which can release the endogenous elicitor from a plant cell wall. Pectinases obtained from plant pathogens hydrolyze the glycosidic bonds of pectin substances in plant cell walls [3, 11]. Van der Heijden *et al.* [14] reported that triterpene was accumulated two- and three-fold over the control cells of *Tabernaemontana divaricata* after treatment with cellulase and pectinase, respectively. Fukui *et al.* [4] explained the existence of an endogenous elicitor by the fact that the addition of pectinase to a cell culture of *Lithospermum erythrorhizon* in a growth medium could induce the formation of shikonin. These studies suggest that menthol production might be enhanced if pectinase were used in a suspension culture of *M. piperita* cells. However, to the best of our knowledge, this is the first report on the elicitation effect of pectinase on a suspension culture of *M. piperita*.

A peppermint cell line was derived from the leaves of *M. piperita* L. Suspended cells of *M. piperita* were established and maintained in a Lin-Staba (LS) medium supplemented with 2 mg 2,4-dichlorophenoxyacetic acid (2,4-D) and 20 g sucrose per liter. Suspension cells were cultivated in shaking incubators at 120 rpm at 27°C with 16 h illumination per day (1,600 lux), and the dry cell weight was 3.8 g/l after cultivation.

Pectinase (EC 3.2.1.15, from *Aspergillus niger*), nigeran, yeast extract, and cellulase (EC 3.2.1.4, from *A. niger*) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Nigeran and yeast extract were sterilized by autoclaving at 121°C for 20 min. The solutions of cellulase and pectinase were sterilized by passing them through a Milex-GS disposable filter unit (pore size, 0.22 μm). Fifty miligram per liter nigeran, 100 mg/l yeast extract, or 50 mg/l cellulase was added into the culture medium as an elicitor. The concentration of pectinase varied from 0 to 25 U/l. The time course of oil production was also analyzed over 15 days. The effectiveness of the elicitor, optimum concentration, and period of elicitation were determined by measuring the specific menthol content, menthol concentration, and contents of major terpenes.

The cell suspension was centrifuged in a 15 ml graduated tube at 1,100×g for 20 min. The centrifuged cells were washed twice with distilled water and dried at 80°C for 24 h to determine the dry cell weight (DCW).

*Corresponding author
Phone: 82-331-290-2585; Fax: 82-331-293-4789;
E-mail: leehyjo@plaza.snu.ac.kr

The essential oil analysis was conducted as follows. The culture medium was collected after centrifugation (2,000×g for 20 min), and the peppermint oleoresin was extracted using a mixture of solution containing pentane and dichloromethane (2:1) for 8 h in a continuous liquid-liquid extractor. Menthone, menthol, and pulegone were analyzed using a gas chromatograph (Hewlett Packard 5890 series II) fitted with a FID detector and Ultra-1 capillary column (Hewlett-Packard, Palo Alto, U.S.A.) packed with 100% dimethylpolysiloxane. The flow rate of the carrier gas was 2 ml/min. Samples were injected at a 25:1 split ratio via an injection port at 250°C with a 1 μl aliquot and a temperature program of 80-150°C at 5°C/min and 150-210°C at 20°C/min. All the data presented represent an average from the results of duplicate experiments.

The effect of the pectinase concentration on the growth of the *M. piperita* cells and menthol production was examined for 5 days. Cell growth was not inhibited by the addition of pectinase up to 20 U/l (data not shown). The maximum menthol concentration (22 mg/l) was obtained with a pectinase concentration of 15 U/l (Fig. 1). Menthol production was decreased at lower concentrations of pectinase (0-10 U/l). Higher concentrations of pectinase (20-25 U/l) had an adverse effect on menthol production. Therefore, the optimum concentration of pectinase for elicitation during menthol production was determined to be 15 U/l. This optimum concentration of pectinase was identical to the concentration (15 U/l) required for the production of shikonin in cell cultures of *L. erythrorhizon* [4]. The time course changes of menthol production at 15 U/l are shown in Fig. 2. The specific menthol content of elicited cells increased up to 27.5 mg/g DCW on day 9, yet

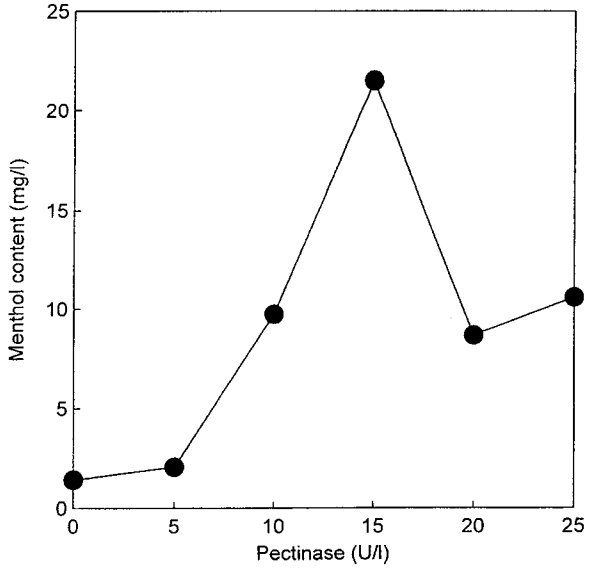

Fig. 1. Effect of pectinase concentration on menthol production using suspension cultures of *M. piperita*.
Initial cell concentration: 0.3 g DCW/l.

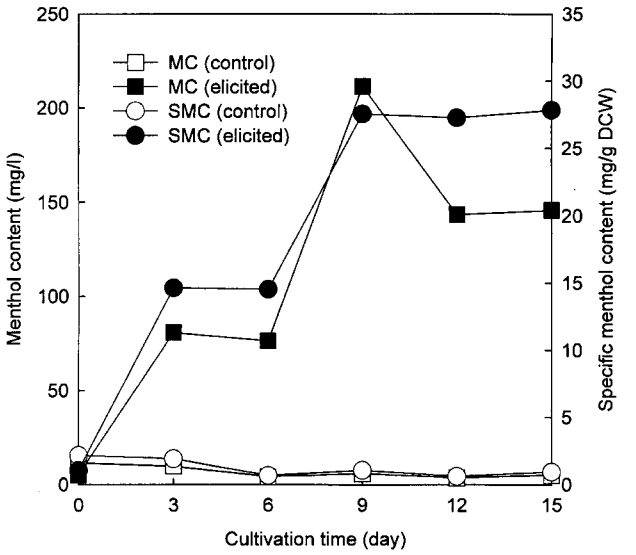

Fig. 2. Effect of pectinase elicitation on menthol production using suspension cultures of *M. piperita*.
Pectinase was treated at 15 U/l. MC, menthol content; SMC, specific menthol content.

that of the control remained at a low level. The changes in menthol concentration followed the same trend as those of specific menthol content. The maximum menthol

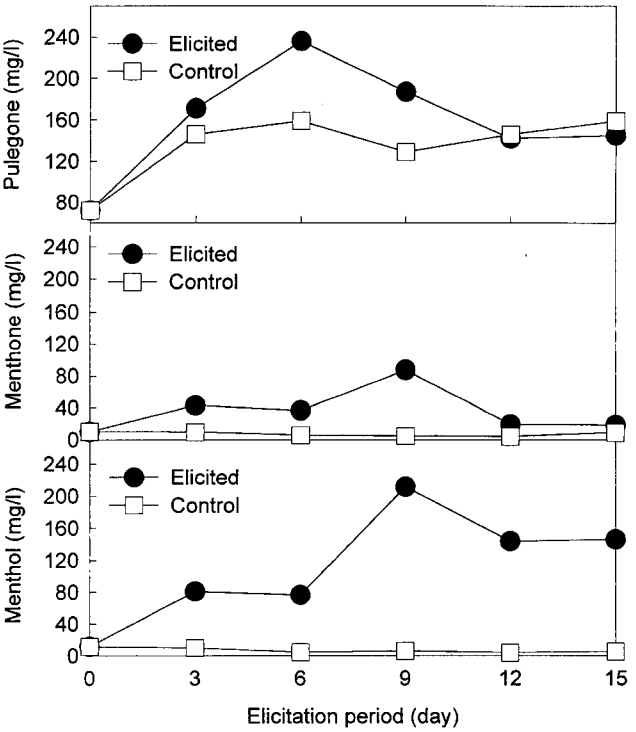

Fig. 3. Effect of pectinase elicitation on the formation of menthol, menthone, and pulegone in suspension cultures of *M. piperita*.
Pectinase was added at a concentration of 15 U/l. ●, Elicited cells; □, non-elicited cells (control).

concentration reached 211.5 mg/l after 9 days of elicitation. Thus, the optimum period for elicitation with pectinase was found to be 9 days during which time menthol production with elicitation was increased 37-fold compared to that of the control.

The time course productions of pulegone, menthone, and menthol are shown in Fig. 3. In elicited cells during the elicitation period, menthol and menthone concentrations were notably increased at the expense of the pulegone concentration. However, in the control without elicitation, the menthol and menthone levels remained low although the pulegone content was high throughout the 15-day period. This result indicates that pectinase elicitation might activate the conversion of pulegone into menthol via menthone, since it is known that pulegone can metabolize to menthone and then to menthol. In our experiments, the content of menthol increased up to 211.5 mg/l after 9 days of elicitation and then decreased. The decreased menthol concentration after 9 days may be caused by the enzymatic degradation of terpenes [5]. Rhodes *et al.* [12] also reported similar results of decreased menthol concentrations in the later stages of shoot cultures of *M. piperita*. In preliminary experiments, four different types of elicitors were tested using a suspension culture of *M. piperita* for the selection of an optimum elicitor: nigeran, yeast extract, cellulase, and pectinase. In terms of menthol production, it was found that pectinase was the best among the elicitors tested. However, although nigeran, yeast extract, and cellulase were suboptimal, they were effective for increasing secondary metabolism in other plant cell cultures [6]. These results are in good agreement with those obtained by Fukui *et al.* [4] regarding the effect of pectinase on secondary metabolite production by *L. erythrorhizon*. The overall conclusion drawn from the results of this study is that pectinase-elicited *M. piperita* cell cultures produce much higher levels of menthol than non-elicited *M. piperita* cell cultures. Pectinase also appears to be a potential elicitor for promoting the menthol biosynthetic pathway.

Acknowledgments

This work was supported by grants from the Korea Science and Engineering Foundation through the Research Center for New Bio-Materials in Agriculture.

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