Journal of Microbiology and Biotechnology
Vol. 6. No. 2, 132-136, 1996

Characteristics of Immobilized Culture of *Mentha piperita* Cells for Oil Production

HA, WON-HO, GUN-JO WOO[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 Foods and Nutrition, Ewha Woman's University, Seoul 120-750, Korea

To investigate the characteristics of immobilized peppermint (*Mentha piperita*) cells, dry cell weight (DCW), change of cell viability, and oil productivity of the immobilized cells were determined. Peppermint cells were immobilized in polyurethane (PU) foams of 5×5×5 mm and cultured in a shaking flask. The maximum DCW was 2.1 mg per foam piece after 20 days of cultivation and the cell density was approximately 420 mg per flask containing 200 foams in 200 ml medium. For the first five days of cultivation, the cell viability was about 80% and decreased to 70% during 5 to 20 days of cultivation. The maximum oil productivity, 148 mg/l was achieved after 40 days of cultivation. The immobilized cells were also cultivated in a bioreactor, equipped with a round spiral type impeller, containing 2,400 PU foams. The cell viability after 30 days of cultivation with chitosan as an elicitor in the bioreactor was 67% and DCW was 2.0 mg per foam piece. Though the cell viability was relatively high in the bioreactor system, the oil productivity was relatively lower than that of the flask system.

Among the food flavors, peppermint oil is one of the most widely used flavors in food industry (16, 21). Many researches have been performed and reported the production of peppermint oil by the cell culture. Lin and Staba (14) tried to produce peppermint oil by the callus culture of *Mentha piperita*. Kim and Lee (11) also reported the production of the peppermint oil, 528 mg/l, in the suspension culture. However, the composition of the oil produced by plant cell culture is different from that of natural peppermint oil. Song and Lee (20) cultivated the peppermint cells in an air bubble bioreactor and observed the production of peppermint oil, 546 mg/l with 22.5% of menthol.

Industrialization of plant cell culture for the production of biochemicals has been limited by low productivity, low growth rate of cells, and high sensitivity to shear. In order to solve these problems various techniques have been tried. The most promising trials are cell immobilization and elicitation (2, 6, 7). Plant cell immobilization technique has been applied for the production of food flavors and colors. It can provide many advantages including re-use of cells, induction of high cell to cell contact, maintenance of stable and active cells for an application to a continuous culture, and increased productivity (3, 4, 13, 15, 18). Furuya *et al.* (9) cultivated immobilized *Coffea arabica* cells for caffeine production in polyurethane (PU) foams for 270 days and observed the maintenance of cell viability and constant caffeine productivity. For the long term cultivation of peppermint cells, the optimal conditions for immobilization of peppermint cells in PU foams were investigated by Kim and Lee (12). More than 90% of peppermint cells were immobilized in PU foams at the optimal conditions. However, the peppermint oil production and cell viability were not measured. Therefore, it should be confirmed whether the immobilized peppermint cells are viable or not and also whether immobilization influences the production of peppermint oil or not.

In this study, immobilization of peppermint cells in PU foams and elicitation to peppermint cells were combined for a semicontinuous culture with high productivity. The growth of cells, change of cell viability, and oil production of the immobilized peppermint cells both in a shake flask and a bioreactor were examined.

MATERIALS AND METHODS

Maintenance of the Peppermint Culture

The callus derived from leaf explant of peppermint (*Mentha piperita* L.) was subcultured on Lin and Staba

*Corresponding author
Key words: *Mentha piperita*, peppermint, immobilized plant cell culture, bioreactor

(LS) medium containing 1.0% agar, 2% sucrose, and 2 mg of 2,4-dichlorophenoxyacetic acid (2,4-D)/l and then cultivated at 27°C under cool white fluorescent light (1600 Lux) for 16 h per day. The pH of medium was adjusted to 5.7 with 1.0 N NaOH before sterilization. Subculture was done every four weeks.

About 2-3 g of fresh callus was transferred to liquid LS medium in a baffled flask (300 ml) for the initiation of peppermint cell suspension culture. The medium was the same as that of callus culture without 1.0% agar. Stock suspension cells were cultivated at 120 rpm and 27°C in a shaking incubator under the same cultivation condition as callus culture. Suspended cells were subcultured in LS liquid medium (200 ml) in a 500 ml shaking flask every four weeks.

Preparation of Polyurethane Foam Matrix

The porocity of PU foams was 145 pore/cm^2 as the optimal condition for immobilization of peppermint cells (12). PU foams were cut into 5×5×5 mm and washed with acetone and absolute ethanol to remove impurites and cytotoxic substances. The washed PU foams were autoclaved at 121°C for 30 min after rewashing with tap water and rinsing with distilled water several times. The PU foams were dried in an oven at 80°C for 48 h prior to use.

Immobilization of Peppermint Cells in PU Foams

Peppermint cells were immobilized as previously described (12) with the following modifications. For the immobilization, stock suspension of peppermint cells cultivated for three weeks was used. Inoculum of 50 ml suspension culture was added to 50 ml of LS medium containing 2 mg of 2,4-D/liter and 200 PU foams in a 300 ml flask. The cells were immobilized at 110 rpm in a shaking incubator under the same conditions of stock suspension culture for six days. Before immobilization, the PU foams were degassed using a spatula and submerged in the medium. Following this procedure, immobilization was performed well without addition of any stabilizing agent, e.g., DEAE-cellulose (12), which may affect the viability of peppermint cells. After immobilization, dry cell weight and viability of immobilized cells were measured.

Immobilized Cell Culture in a Shaking Flask

Peppermint cells immobilized in PU foams for six days were transferred to a 500 ml shaking flask containing 200 ml of fresh LS medium. Foams were collected every five days to measure the viability and dry cell weight (DCW) of immobilized cells.

Immobilized Cell Culture in a Bioreactor

A bioreactor which has a round spiral paddle type impeller was manufactured for immobilized *Mentha piperita* cell culture. A modification of impeller was necessary for plant cell cultivation because of sensitivity of plant cells to mechanical shear (17).

To fill 30% of the total volume of the reactant, degassed 2,400 foams were added to the medium (1.6 l) in the 2.0 liter bioreactor. After sterilization, 400 ml of 21 day old cell suspension culture was inoculated to the medium. After immobilization for 10 days, immobilized cells were cultivated by exchanging the medium with fresh medium containing chitosan (5.0 mg/ liter of medium). The culture medium was collected and refilled with 200 ml of new medium by 10 day intervals to monitor the oil production. After 30 days of cultivation, the final DCW and cell viability were measured.

Preparation of Elicitor and Elicitation

Chitosan (1.0 g) purchased from Sigma Chemical Co. was dissolved by stirring overnight in 90 ml of 0.1 N acetic acid (22). After dissolving, centrifugation was done at 27,000×g for 20 min to eliminate insoluble impurities. Then, the pH of the supernatant was adjusted to 8.0 with 5.0 N of NaOH. The precipitate was washed with distilled water by vortexing and freeze dried. For elicitation, chitosan (5.0 mg/liter of medium) was added to the medium at the initiation of cultivation.

Analysis of Cell Concentration and Cell Viability

For the measurement of DCW, the immobilized foams were washed with 10 ml distilled water and filtered through a Whatman No. 2 filter paper. The foams were dried at 80°C for 24 h and weighed. DCW was determined by subtracting the mean weight of preweighed empty foam from that of immobilized foam.

Cell viability was measured by 2,3,5-triphenyl tetrazolium chloride (TTC)-reducing reaction (10). Ten foams of immobilized peppermint cells were collected to measure DCW and cell viability. Five foams were taken from the medium to an oven maintained at 80°C and then DCW was determined. The others were incubated in 10 ml of 0.1 N Tris buffer (pH 9) with 50 mg of TTC for 12 h at 27°C under dark condition. For the exraction of red formazan, the PU foams were transferred to 3 ml of 95% ethanol and heated at 60°C for 15 min. Then, the foams were centrifuged and the supernatant was used to measure absorbance at 485 nm using 95% ethanol as a blank. Cell viability was determined by comparing with the standard curve for peppermint cells.

Analysis of Peppermint Oil

Peppermint culture medium was filtered and cells were removed for extraction. The peppermint oil in the filtered medium was extracted by a continuous liquid-liquid extractor at 45°C for 12 h with the mixture of pentane-dichloromethane (2 : 1, v/v) as solvent (11). Extracted sample was purified by passing through a column packed in a bilayer with anhydrous sodium sulfate and active charcoal powder so as to remove water and other impurities. Concentration was done using a rotary vacuum evaporator and the content of essential oil was determined.

Regression Analysis

A best fit model to predict productivity was developed using stepwise regression. Selection of the best fit model was based on R-sguare, probability, and residuals vs predicted value.

RESULTS AND DISCUSSION

Growth of Immobilized Peppermint Cells

The relationship between cultivation time of immobilized peppermint cells and DCW was determined. As presented in Fig. 1, immobilized cells grew in PU foams for the first five days. DCW was not increased significantly after 5 days of cultivation. When the immobilization was finished, DCW was only 1.6 mg/foam. The maximum DCW reached 2.1 mg/foam after 20 days of cultivation. In a flask containing 200 foams and 200 ml LS medium, cell density was about 420 mg/flask. This value was shown to be lower due to a possible environmental change around cells during immobilization than that of peppermint cell suspension culture, which was about 700 mg/flask (5).

Change of Cell Viability

The effect of immobilization on the cell viability during immobilized cell culture was shown in Fig. 2. It shows the increase of cell viability for the first five days after the initiation of immobilized cell culture, and then the viability decreased slowly after five days of cultivation. The cells seemed to be damaged by immobilization, because the cell viability was about 80% compared to the beginning of cultivation. For the first five days, the cell viability increased to some extent. This increase may be due to the growth of viable cells and the lysis of dead cells. The cell viability slowly decreased to 70% during five to 20 days of cultivation. The results were similar to those of immobilized culture of *Botryococcus braunii* cells in PU foams, which showed a serious decrease in cell viability to less than 30% (1). However, the results in Fig. 2 showed that the immobilized cells could maintain high stability to an extended period of cultivation. Kim and Lee (12) reported the deterioration of immobilized peppermint cells with a stabilizing agent, DEAE-cellulose. Immobilization without a stabilizing agent, with degassed PU foams, and with increased inoculum were the key factors to maintain the high cell viability in this study (Fig. 2).

Time Course of Oil Production in Immobilized Cell Culture of Peppermint Cells

To determine the optimum cultivation period of immobilized peppermint cells, the oil productivity at 20, 40, and 60 day was analyzed (Fig. 3). As shown in Fig. 3, oil production reached maximum level after 30 to 40 days of cultivation. The maximum productivity was 148 mg/l at 40 day of cultivation. After 40 days of cul-

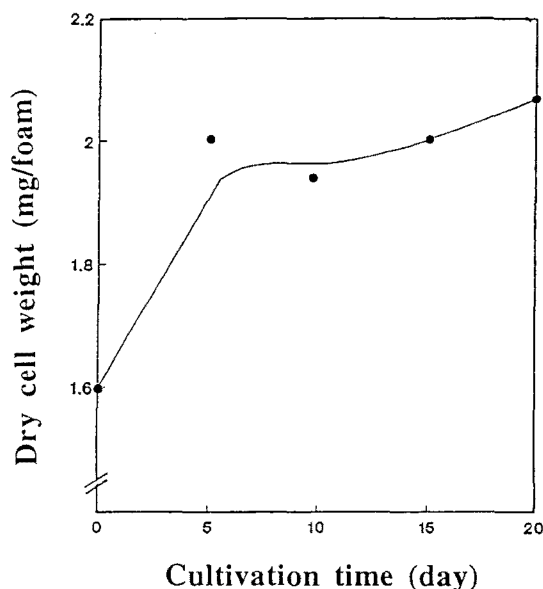

Fig. 1. Change of dry cell weight in immobilized culture of peppermint cells.

The cells were immobilized in polyurethane foams for six days. The immobilized cells were incubated at 27°C with 16 h illumination of 1600 Lux/day and transferred to a 500 ml shaking flask containing 200 ml of fresh LS medium.

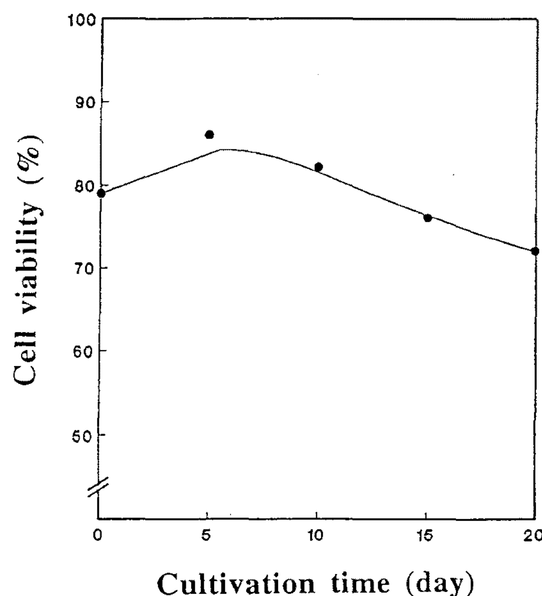

Fig. 2. Change of cell viability in immobilized culture of peppermint cells in the LS medium.

The cells were immobilized in polyurethane foams for six days. The immobilized cells were incubated at 27°C with 16 h illumination of 1600 Lux/day and transferred to a 500 ml shaking flask containing 200 ml of fresh LS medium.

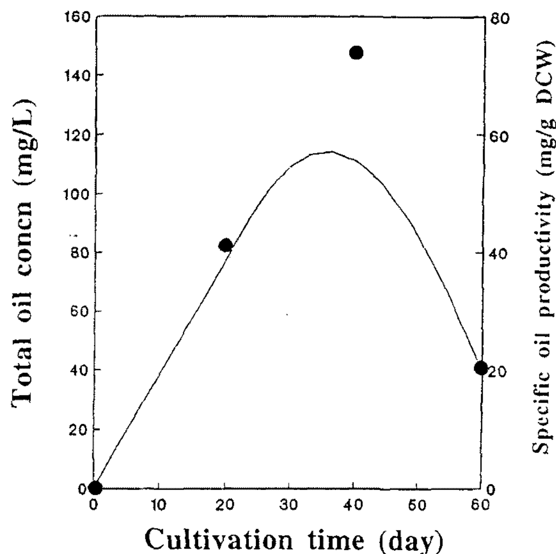

Fig. 3. Time course of oil productivity in immobilized culture of peppermint cells.

The cells were immobilized in polyurethane foams for six days. The immobilized cells were incubated at 27°C with 16 h illumination of 1600 Lux/day and transferred to a 500 ml shaking flask containing 200 ml of fresh LS medium.

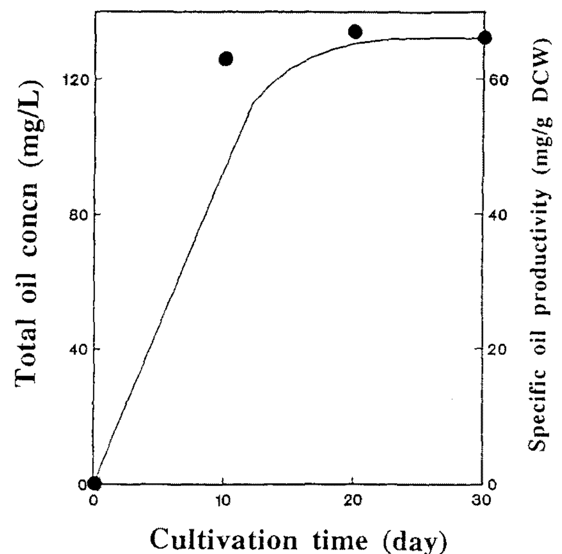

Fig. 4. Total and specific oil productivity in immobilized culture of peppermint cells in a bioreactor with chitosan as an elicitor.

The cells were immobilized in polyurethane foams for ten days in the bioreactor. Chitosan (5.0 mg/l of medium) was added to the medium at the initiation of cultivation. To fill 30% of the total volume of the reactant, degassed 2,400 foams were added to the medium (1.6 l) in the 2.0 l bioreactor.

tivation, the oil productivity decreased rapidly to nearly one fourth times of the maximum productivity. This trend was also observed in the suspension culture of *Mentha piperita* (5). It can be postulated that immobilized peppermint cells and suspended peppermint cells have a similar tendency for oil production, but the suspension culture system reached to the maximum productivity four times faster than immobilized culture system (5). Total oil concentration during immobilized peppermint cells was lower than that of the suspended cells (5). This phenomena were also observed in the immobilized culture of *Thalictrum rugosum* (8). In the case of immobilized culture of *T. rugosum* cells in glass fiber matrix, the maximum productivity of columbamine and berberine was decreased by about 50% and 30%, respectively.

However, the specific oil productivity of immobilized peppermint cell culture (See the right Y axis in Fig. 3) was similar to that obtained in the suspension culture (5). Therefore, if cultivated with higher immobilized cell density, the total oil amount could be increased to the same level as that of suspended peppermint cell culture.

Immobilized Culture of Peppermint Cells in a Bioreactor with Elicitation

To investigate the characteristics of oil production during immobilized culture with elicitation in a semi-perfusion culture system, the peppermint oil productivity of the culture at 10, 20, and 30 day was analyzed (Fig. 4). As presented in Fig. 4, total oil concentration and specific oil productivity were lower than those in a shaking flask culture (Fig. 3). This tendency was similar to other plant cell culture in a bioreactor. In *Catharanthus roseus* suspension culture, serpentine formation was very low when grown in any of the bioreactor types compared to suspension culture (19).

The cell viability after 30 days of cultivation was 67% and DCW was 2.0 mg/foam. The reasons for the low productivity of peppermint oil with high viability could not be elucidated clearly. However, different culture conditions between the flask and the bioreactor culture, e.g., rheological properties of the culture medium, the intensity of illumination, and agitation effect might affect the productivity of peppermint oil.

Acknowledgements

This work was supported by grants from Korea Science and Engineering Foundation and Research Center for New Biomaterials in Agriculture.

REFERENCES

1. Baillez, C., C. Largeau, E. Casadevall, L. W. Yang, and C. Berkaloff. 1988. Photosynthesis, growth and hydrocarbon production of *Botryococcus braunii* immobilized by en-

trapment and adsorption in polyurethane foams. *Applied Microb. Biotechnol.* **29**: 141-147.

2. Brodelius, P. and K. Mosbach. 1982. Immobilized plant cells: general aspects. *J. Chem. Tech. Biotechnol.* **32**: 330-337.
3. Brodelius, P. 1984. Immobilization of cultured plant cells and protoplasts. p. 535. *In* I. D. Vasil (ed.). *Cell Culture and Somatic Cell Genetics of Plants.* vol **1**, Academic Press, New York.
4. Brodelius, P. 1986. Immobilized plant cells and protoplasts. p. 287. *In* P. V. Ammirato, D. A. Evans, W. R. Sharp, and Y. Yamada (eds.). *Handbook of Plant Cell Culture*, vol. **4**, Macmillan Publishing Company, New York.
5. Chang, J. H. 1993. Effect of elicitation on the production of peppermint oil during suspension culture of *Mentha piperita* L.. Master Thesis, Seoul National University, Seoul, Korea.
6. Eliert, U., B. Welters, and F. Constabel. 1986. Elicitor-stimulation of monoterpene indole alkaloid formation in suspention culture of *Cantharanthus roseus. J. Plant Physiol.* **126**: 11-22.
7. Eliert, U. 1987. Elicitation: methodology and aspects of application. p. 153. *In* I. D. Vasil (ed.). *Cell Culture and Somatic Cell Genetics of Plants*, vol **4**, Academic Press, New York.
8. Facchiini, P. J. and F. DiCosomo. 1991. Plant cell bioreactor for the production of protoberberine alkaloids from immobilized *Thalictrum rugosum* cultures. *Biotechnol. Bioeng.* **37**: 397-403.
9. Furuya, T., K. Koge, and Y. Orithara. 1990. Long term culture and caffeine production of immobilized coffee (*Coffea arabica*) L. cells in polyurethane foam. *Plant Cell Reports* **9**: 125-130.
10. Iborra, J. L., J. Guardiola, S. Montaner, M. Canovas, and A. Manjon. 1992. 2,3,5-Triphenyltetrazolium chloride as a viability assay for immobilized plant cells. *Biotechnol. Tech.* **6**: 319-322.
11. Kim, J. H. and H. J. Lee. 1992. Production of monoterpenoid flavor compounds by suspension culture of peppermint cells. *J. Korean Agric. Chem. Soc.* **35**: 443-448.
12. Kim, Y. S. and H. J. Lee. 1993. Optimal conditions for the immobilization of peppermint cells in polyurethane foam matrix. *Foods Biotechnol.* **2**: 141-145.
13. Knorr, D., S. M. Miazga, and R. A. Teutonico. 1985. Immobilization and permeabilization of cultured plant cells. *Food Technol.* **39**: 135-140.
14. Lin, M. L. and E. J. Staba. 1961. Peppermint and spearmint cultures. I. Callus formation and submerged culture. *Lloydia* **24**: 139-144.
15. Lindsey, K. and M. M. Yeoman. 1983. Novel experimental system for studying the production of secondary metabolites by plant tissue cultures. p. 39-66. *In* H. Smith (ed.). *Soc. Exp. Biol. Sem. Ser. Mantell, Plant Biotech.* vol. **18**, Cambridge Univ. Press.
16. Mulder-Krieger, T. H., R. Verpoorte, A. B. Svendsen, and J. J. C. Scheffer. 1988. Production of essential oils and flavours in plant cell and tissue cultures. *Plant Cell, Tissue, and Organ Culture* **13**: 85-90.
17. Panda, A. K., S. Mishra, V. S. Bisaria, and S. S. Bhojwani. 1989. Plant cell reactors-a perspective. *Enzyme Microb. Technol.* **11**: 386-397.
18. Roservear, A. and C. A. Lambe. 1985. Immobilized Plant Cells. *Adv. Biochem. Eng.* **31**: 36-57.
19. Scragg, A. H., P. Morris, E. J. Allan, P. Bond, and M. W. Fowler. 1987. Effect of scale-up on serpentine formation by *Catharanthus roseus* suspension culture. *Enzyme Microb. Technol.* **9**: 619-624.
20. Song, E. B. and H. J. Lee. 1993. Characteristics of growth and oil production of peppermint cells in an air-bubble reactor. *Korean J. Biotechnol. Bioeng.* **8**: 495-503.
21. Whitaker, R. J. and H. Hashimoto. 1986. Production of secondary metabolites, p. 264. **In** P. V. Ammirato, D. A. Evans, W. R. Sharp, and Y. Yamada, (eds.). *Handbook of Plant Cell Culture*, vol. **4**, Macmillan Publishing Company, New York.
22. Young, D. H., H. Kohle, and H. Kauss. 1982. Effect of chitosan on membrane permeability of suspension-cultured *Glycine max* and *Phaselous vulgaris* cells. *Plant Physiol.* **70**: 1449-1454.

(Received February 22, 1996)