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Improved Production of Essential Oils by Two-phase Culture of *Mentha piperita* cells

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In cultures of *Mentha piperita*, it has been reported that some cell lines can synthesize essential oils[1]. Some efforts have been made to produce essential oil in callus or cell suspension culture[2,3] and the effects of many culture conditions such as carbon source, exposure time to light, initial seeding density, pH, agitation, hormone concentration, and operating mode and type of bioreactor on cell growth and essential oil formation have been investigated[4-6]. However, some problems still remain unresolved in improving peppermint oil formation.

In many cases, plant cell suspensions yield secondary metabolites at a very low level. The low yield of secondary metabolites during conventional single phase culture may be enhanced by the introduction of a second phase. Up to the present, a very limited number of second phases have been tried experimentally and no general rule about the influence on plant cells can be formulated[7-9]. Recently, the second phase of LiChroprep RP-8(RP-8), a silica gel with outer SiOH group covalently bounded to C_8 hydrocarbons, has been successfully employed to select monoterpene producing cell lines[10]. Also, RP-8 has been employed as a second phase in suspension culture of *Valeriana wallichii* to accumulate valepotriates[11]. These previous studies suggest that use of RP-8 may create the possibility to enhance secondary metabolite production from *M. piperita* cell culture. Therefore, we have attempted to examine the feasibility of RP-8 for enhancing essential oil production from *M. piperita* cell culture.

In this study, the effect of two-phase culture on *M. piperita* cell growth and essential oil formation is investigated in shake flasks.

Peppermint cell line was derived from the leaves of *M. piperita*. The basic medium was Lin-Staba(LS) medium supplemented with 0.2 mg 2, 4-dichlorophenoxyacetic acid and 20 g sucrose per liter. The cells were subcultured every 12 days into 200 m*l* of baffled Erlenmyer flasks containing 50 m*l* of the liquid medium and incubated at 27°C in the white fluorescent light for 16 hrs per day on a gyratory shaker at 100 rpm. The initial pH of the medium was adjusted 5.7 before autoclaving.

For shake flask culture operation, 200 m*l* and 500 m*l* baffled shake flasks with 50 m*l* and 160 m*l* of medium were used. The temperature was maintained at 27°C and agitation speed was 100 rpm.

For two-phase culture, 200 m*l* and 500 m*l* baffled shake flasks with 50 m*l* and 160 m*l* of medium and a membrane bag containing a second phase were used. The solid second phases used were XAD-4(Sigma), XAD-7(Sigma) and RP-8(Merck). XAD-4 and XAD-7 were pretreated prior to use as the procedure described elsewhere[8]. RP-8(40-63 μm) was washed with several volumes of water before use. When the cells were treated with RP-8, a membrane bag containing 0.5% RP-8 was immersed in the culture broth for 2 days and then the membrane bag was removed from the

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broth, unless specified otherwise.

For combined two-phase culture with chitosan elicitation, 500 m*l* baffled shake flasks with a working volume of 160 m*l* were used for the experiments. For each RP-8 treatment, the cells were treated with RP-8 for 2 days in the way identical to that described above. For each elicitation step, chitosan was added to make 100 mg/L chitosan to the medium of suspension culture.

The cell suspension was centrifuged in a 15 m*l* graduated tube at 1100 g for 20 min. and the percentage of cell volume after centrifugation was determined as the packed cell volume (% PCV). A specific growth rate was estimated at an exponential phase of cell growth. Essential oil analysis is as follows: cells were separated from culture broth after filtration. Cells were washed with dd H_2O, and homogenized by the homogeniger at 20000 rpm for 10 min. Cells or culture broth was extracted for 2 hrs using pentane-dichloromethane 2 : 1 (v/v) in order to analyze intracellular oil or extracellular oil content. Essential oil content was measured by a gas chromatograph (GC) equipped with a flame ionized detector. The GC conditions are as follows: injection volume, 1 μl; fused silica capillary column coated with SE-30, 15 m×0.54 mm I.D.; oven temperature 80°C (2 min.), 80°C~200°C (at the increasing rate of 5°C/min.), 200°C~240°C at (20°C/min.), 240°C (3 min.). Oil component identification was conducted by comparing retention time of the authentic compounds such as isomenthol, menthol, menthone, menthylacetate and pulegone. The content of separate components was determined using camphor as an internal standard.

Several runs were made to see if a second phase affects *M. piperita* cell culture. XAD-4, XAD-7 and RP-8 were added at the start of the culture to 50 m*l* LS medium in 200 m*l* flasks containing *M. piperita* cells. The cell growth rates were 0.24, 0.22, 0.21, 0.17, 0.13, 0.11 day^{-1} in the medium containing 0.5% RP-8, 1% RP-8, 0.5% XAD-4, 1% XAD-4, 0.5% XAD-7 and 1% XAD-7, respectively. The cell growth rate in the medium containing 0.5% or 1% RP-8 was better than that in the medium containing XAD-4 or XAD-7. Treatment with 0.5% RP-8 gave the best growth rate. This result indicates that the addition of solid second phases affected *M. piperita* cell growth depending on the type and concentrations employed.

Experiments were carried out to study the effects of RP-8 on essential oil production. Cells were grown in LS media for 7 days and then treated with RP-8 for 2 days. Thereafter the cells were incubated for 11 days. Two-phase culture did not impair *M. piperita* cell growth (**Fig. 1**). Peppermint oil contents of 89 and 118 μg/L were obtained in the control and two-phase culture runs, respectively. RP-8 treatment enhanced essential oil formation 32%. Although the reasons for

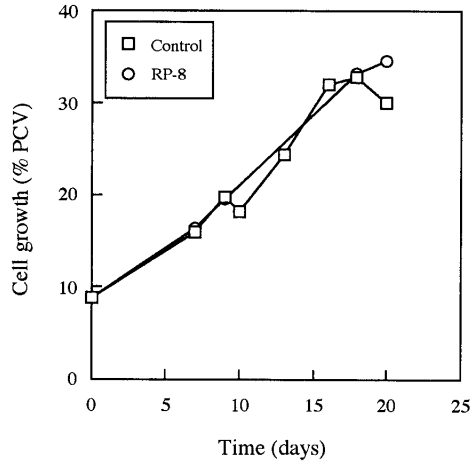

Fig. 1 The growth kinetics in two-phase culture of *M. piperita* cells.

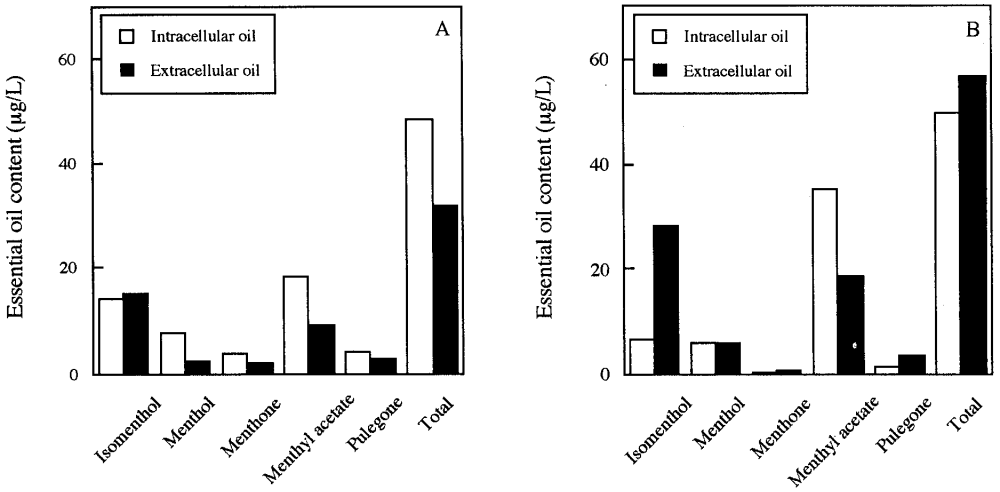

Fig. 2 Effect of LiChroprep RP-8 on excretion of essential oil in *M. piperita* cell culture.
A, before RP-8 treatment; B, after RP-8 treatment

this enhancement are not known, it is possible that RP-8 adsorbs inhibitors of secondary metabolism towards essential oil biosynthesis. It is interesting to note that a significant accumulation of menthyl acetate was detected from the GC analysis of RP-8 extract (Data not shown).

As shown in **Fig. 2**, an increase occurred in the total content of extracellular oil after RP-8 treatment. Evidently, addition of RP-8 to the culture not only enhanced total oil level, but increased somewhat excretion of essential oils. This result suggests that the presence of a second phase may be capable of altering metabolism of these cells toward biosynthesis and excretion of essential oils.

Improved productivity has been reported for *M. piperita* cells subjected to chitosan elicitation[12]. Thus, two-phase culture may be combined with chitosan elicitation to enhance essential oil formation. To verify this point, the runs of different processes such as RP-8 treatment only, RP-8 treatment elicitation and elicitation RP-8 treatment were made. For the run of RP-8 treatment

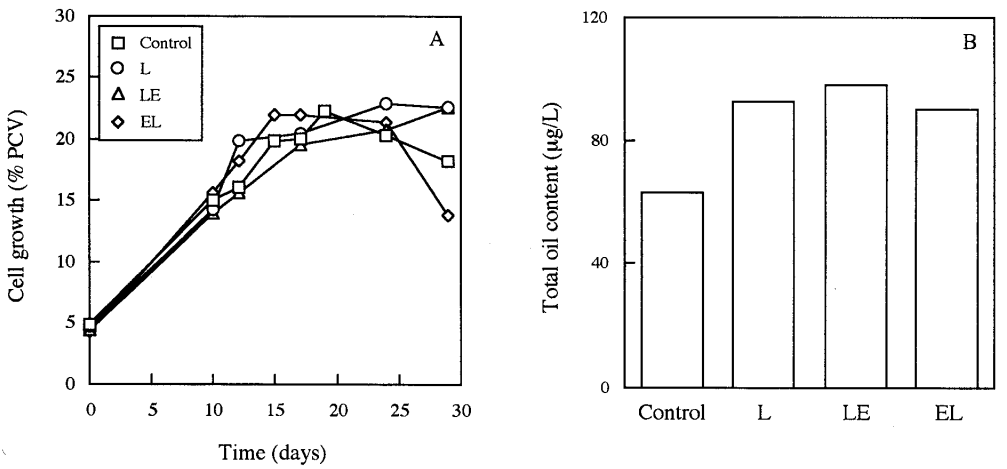

Fig. 3 *Mentha piperita* cell growth (A) and total oil formation (B) in combined two-phase culture with elicitation.
In the run of L, the cells were treated with RP-8 for 2 days and incubated for 17 days. In the run of LE, the cells were treated with RP-8 for 2 days, elicited with chitosan for 5 days, and incubated for 10 days. In the run of EL, the cells were elicited with chitosan for 5 days, treated with RP-8 for 2 days, and then incubated for 10 days.

only(L), 10-day-cultured cells were treated with RP-8 for 2 days and then incubated for an additional 17 days. In the run of RP-8 treatment-elicitation(LE), 10-day-cultured cells were treated with RP-8 for 2 days and then elicited with chitosan for 5 days. Thereafter, the cells were incubated for an additional 10 days. For the run of elicitation-RP-8 treatment(EL), 10-day-cultured cells were elicited with chitosan for 5 days and then treated with RP-8 for 2 days. Then, the cells were incubated for an additional 10 days. As shown in **Fig. 3**, the final PCV in either L or LE process was higher than that of EL or the control, whereas the total oil content in the process of L, LE, or EL was higher than that of the control. In total oil content LE process was best among the processes tested. For this process the level of total oil content was improved 55%, compared to the control. It is also observed that elicitation step in either EL or LE process did not increase the level of oil production much, compared to the L process. Evidently, combined use of RP-8 and chitosan elicitor was not synergistic. Hence, it can be concluded that elicitation process by chitosan is less effective. One of the possible explanations is that the second phase, RP-8 also elicits *M. piperita* cells and thus the effect of chitosan treatment is insignificant.

It is necessary to investigate further operating parameters which may affect RP-8 addition for improved oil formation in *M. piperita* cell culture. However, our preliminary data suggest that the use of a second phase such as LiChroprep RP-8 may be an attractive means of improving *M. piperita* cell growth and essential oil production.

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