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Enhanced Essential Oil Formation by Two-phase Culture of *Mentha piperita* Cells in Shake Flask and Air-lift Bioreactors

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Effect of two-phase culture on *Mentha piperita* cell growth and essential oil formation was investigated using shake flask and air-lift bioreactors. LiChroprep RP-8(RP-8) addition did not impair *M. piperita* cell growth, but resulted in stimulated formation of essential oils and increased ratios of extracellular oil to intracellular oil formation. However, the combined use of RP-8 and chitosan elicitor was not synergistic. Volumetric productivity of essential oils in RP-8 treated culture using cell-recycled air-lift bioreactor was 6.9 μg/l · day which was substantially higher than that obtainable from the control. Our results demonstrate the potential of a second phase to enhance overall productivity for *M. piperita* cell culture.

In cultures of *Mentha piperita,* it has been reported that some cell lines can synthesize essential oils (8). Some efforts have been made to produce essential oil in callus or cell suspension culture (9, 14) 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 (2, 6, 7, 10-12). However, some problems still remain unresolved in improving the peppermint oil formation.

In many cases, plant cell suspensions yield secondary metabolites at 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 has been tried experimentally and no general rule about the influence on plant cells can be formulated (4, 13, 15). 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 (3). Also, RP-8 has been employed as a second phase in suspension culture of *Valeriana wallichii* to accumulate valepotriates (1). 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 flask and air-lift bioreactors.

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Key words: *Mentha piperita* cells, LiChroprep RP-8, essential oil formation

MATERIALS AND METHODS

Cell Line and Seed Culture Preparation

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 ml of baffled Erlenmyer flasks containing 50 ml of the liquid medium and incubated at 27°C in the white fluorescent light for 16 h per day on a gyratory shaker at 100 rpm. The initial pH of the medium was adjusted 5. 7 before autoclaving.

Shake Flask Culture and Batch Air-lift Bioreactor Operation

200 ml, 500 ml and 1 liter baffled shake flasks with 50 ml, 160 ml, and 160 ml of medium were used for the experiments. The temperature was controlled at 27 °C and agitation speed was 100 rpm. Bioreactor used was a 1 liter external loop air-lift bioreactor with a

working volume of 0.68 liter. The temperature was maintained at 27°C and the aeration rate was 0.1 vvm.

Two-phase Culture

200 ml and 1 liter baffled shake flasks with 50 ml and 160 ml of medium and a membrane bag containing a second phase were used for the experiment. 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 (13). RP-8 (40-63 μm) was washed with several volumes of water before use. When the cells were treated with RP-8 in shake flasks, 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 broth, unless specified otherwise. In an air-lift bioreactor, RP-treatment was done for 24 h by immersion of a membrane bag containing 0.5% RP-8.

Combined Two-phase Culture with Chitosan Elicitation

500 ml baffled shake flasks with a working volume of 160 ml 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 shake flask runs. For each elicitation step, chitosan was added to make 100 mg/l chitosan to the medium of suspension culture.

Cell-recycled Air-lift Bioreactor Operation

The cell-recycled air-lift bioreactor culture was basically the same as that of a batch air-lift bioreactor with an additional provision for continuous substrate delivery and waste withdrawal. Cell-recycle was done by natural sedimentation of *M. piperita* cells within a cell settler placed vertically inside the bioreactor. The dimensions of glass settler for external loop air-lift bioreactor was 2.5 cm×15.0 cm (diameter×height). Other conditions and medium composition were the same as those described for batch culture.

Biomass and Essential Oil Analyses

The cell suspension was centrifuged in a 15 ml 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. A growth index was calculated as

$$\frac{\text{Final PCV} - \text{Initial PCV}}{\text{Initial PCV} \times \text{Culture time (day)}}$$

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 h using pentane-dichloromethane 2:1 (v/v) in other 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), 80~200°C (at the increasing rate of 5°C/min), 200~240°C at (20°C/min), 240°C (3 min).

RESULTS AND DISCUSSION

Effect of Type of Second Phases on *M. piperita* Cell Growth

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 ml LS medium in 200 ml flasks containing *M. piperita* cells. As shown in Fig. 1, 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, and that RP-8 addition did not interfere with *M. piperita* cell growth.

Effect of RP-8 Addition Time on *M. piperita* Cell Growth

RP-8 was added to the medium at 0, 4, 7, 10 and

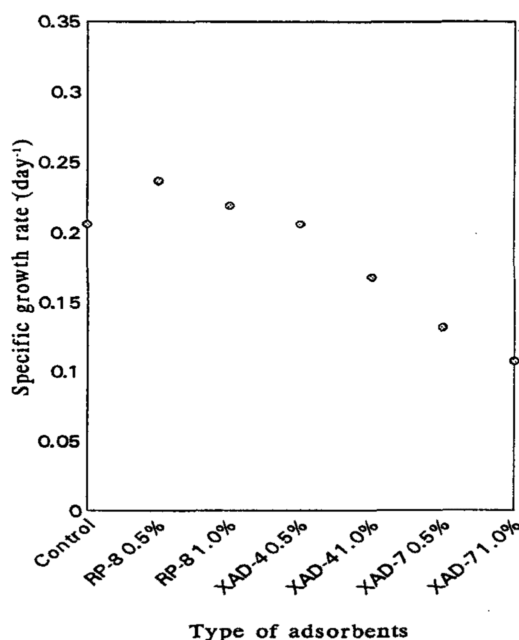

Fig. 1. Effect of type of second phases on *M. piperita* cell growth.

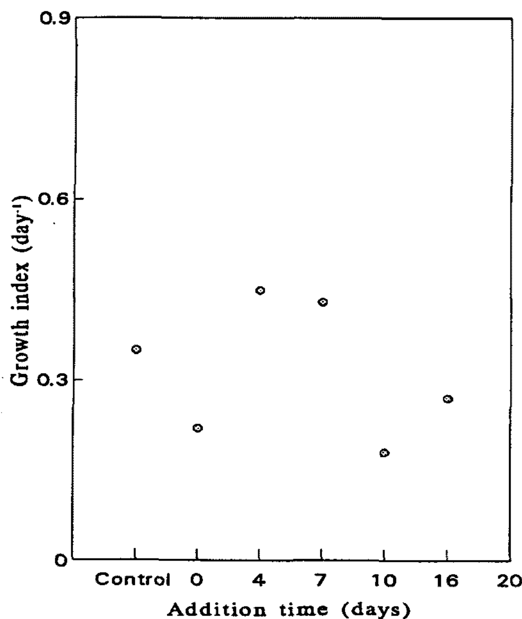

Fig. 2. Effect of addition time of LiChroprep RP-8 on *M. piperita* cell growth.

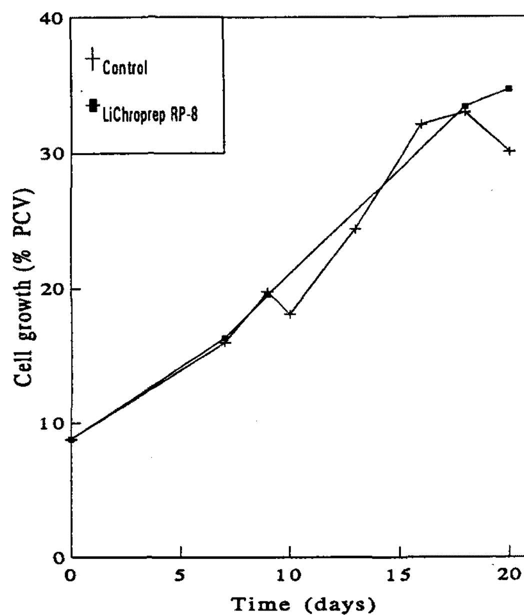

Fig. 3. The growth kinetics in two-phase culture of *M. piperita* cells.

16 days after the culture to see if addition time of a second phase affects *M. piperita* cell culture. As shown in Fig. 2, cell growth index was higher when RP-8 was added to the medium at 4 or 7 days after the culture, whereas the index was lower when RP-8 was added at 0, 10, 16 days after the culture. This reduction of cell growth index may be due to more loss of essential components from the medium with an earlier addition of RP-8 or the presence of growth-inhibitory components at higher level in the medium with a later addition of RP-8. It is clear that the growth response of *M. piperita* cells to a second phase depends on addition time of RP-8 to the culture.

Two-phase Culture Kinetics

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 7 days. Then, the cells were treated with RP-8 for 2 days again and incubated for additional 7 days. As shown in Fig. 3 and 4, RP-8 treatment did not impair *M. piperita* cell growth, but enhanced essential oil formation. Although the reasons for 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). This indicates that menthyl acetate may be one of the most important inhibitors to secondary metabolism of essential oil biosynthesis.

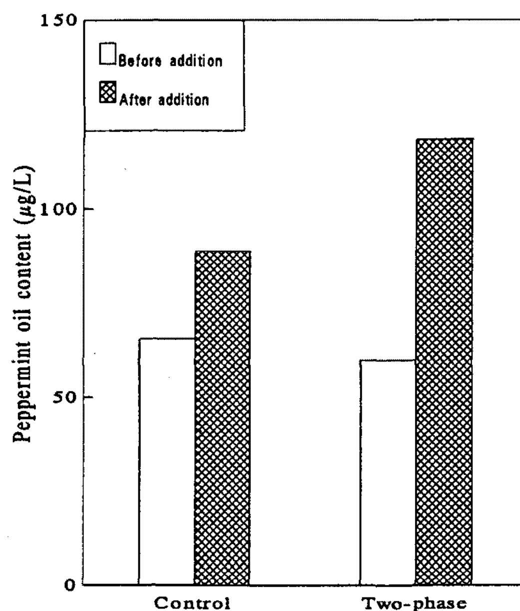

Fig. 4. Essential oil production in two-phase culture of *M. piperita* cells.

As shown in Fig. 5, a significant increase occurred in ratios of extracellular menthol and isomenthol relative to intracellular content after each RP-8 treatment. Evidently, addition of RP-8 to the culture not only enhanced total oil level, but increased excretion of essential oils. This result suggests that the presence of a second phase may be capable of altering metabolism

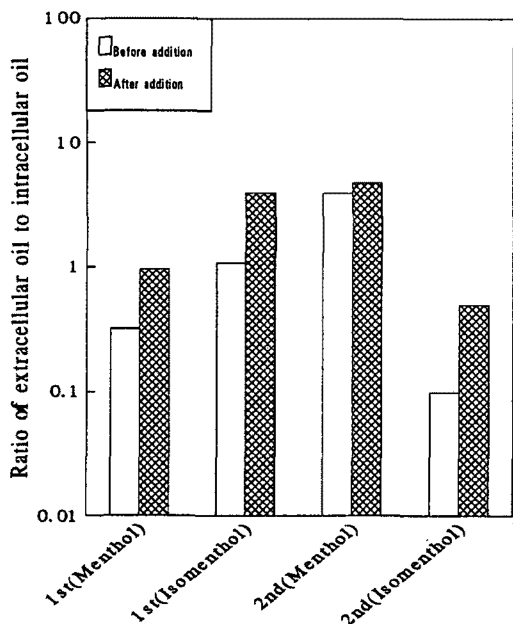

Fig. 5. Effect of LiChroprep RP-8 on excretion of essential oil in *M. piperita* cell culture.

of these cells toward biosynthesis and excretion of essential oils.

Combined Two-phase Culture with Elicitation

Improved productivity has been reported for *M. piperita* cells subjected to chitosan elicitation (5). 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 only (L), 10-day-cultured cells were treated with RP-8 for 2 days and then incubated for 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 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. Thereafter, the cells were incubated for an additional 10 days. As shown in Fig. 6 and 7, 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,

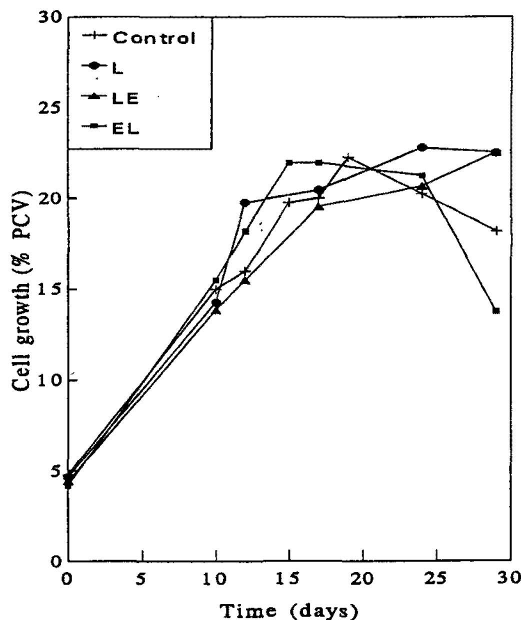

Fig. 6. *Mentha piperita* cell growth in combined two-phase culture with elicitation.

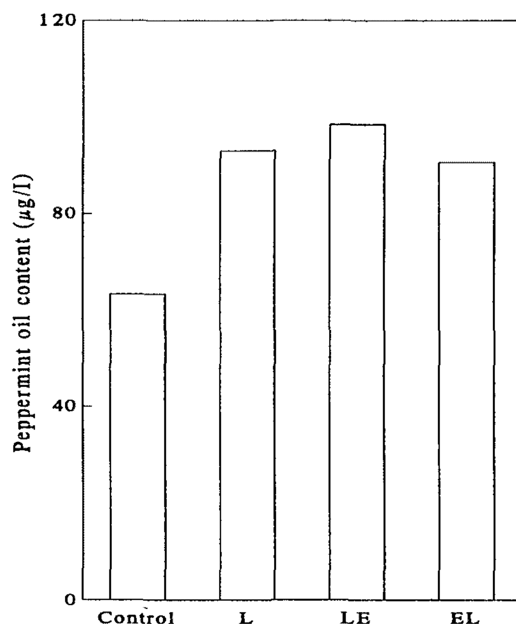

Fig. 7. Essential oil production in combined two-phase culture with elicitation.

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.

Effect of LiChroprep RP-8 on Cell-recycled Air-lift Bioreactor Culture

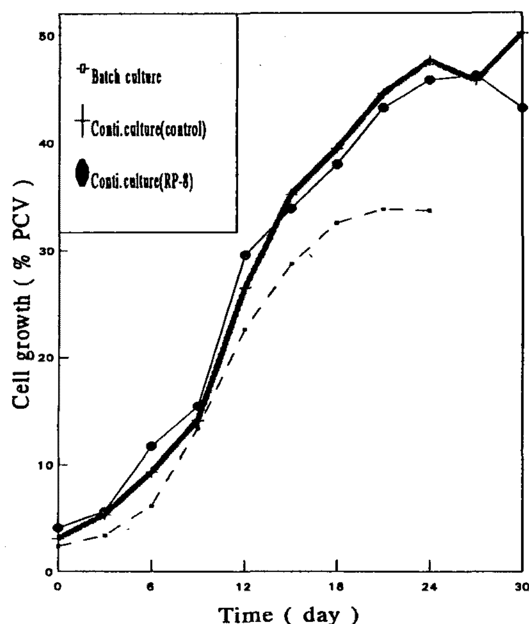

Fig. 8. Effect of LiChroprep RP-8 on cell-recycled air-lift bioreactor culture.

Table 1. Effect of RP-8 on oil productivity in cell-recycled air-lift bioreactor.

Run	Productivity (μg/l · day)
Control	4.7
RP-8 treated	6.9

Mentha piperita cells were grown batchwise in two external-loop bioreactors with working volume of 0.68 liter for 9 days. Thereafter cell recycle operations were started applying the dilution rate of 0.27 day^{-1}. RP-8 treatment was intermittently employed in one of two simutaneous runs of cell-recycled continuous culture to examine the dynamics of cell growth and essential oil formation. Each treatment was done for 24 h by immersion of a membrane bag containing RP-8. As shown in Fig. 8, RP-8 treatment did not impair *M. piperita* cell growth. This result is consistent with those obtained from two-phase culture of *M. piperita* cells in shake flasks. At about 24th day, cell-recycled continuous culture reached a quasi-steady state, which was within four residence times from the start up of cell-recycle operations. The peppermint oil productivity at quasi-steady state was taken as a representative value for each run. As shown in Table 1, volumetric productivity of essential oils in RP-8 treated culture was 47% higher than that obtainable from the control. These results led to the conclusions that the addition of a lipophilic phase, RP-8 to the culture medium can be helpful for secondary metabolite accumulation in *M. piperita* cell culture.

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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