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Efficient Production of Useful Metabolites Using Protoplasts

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Introduction

Many useful metabolites produced by plant and microbial cells which are stored within the cells thus making their efficient production very difficult. The amounts and rates of production of these natural products are still very low. Moreover, the cells must be disrupted in order to extract and purify the desired product. These add to both the complexity of the process and the production cost (particularly, in plant cells, these problems limit their industrial use). Various methods for inducing release of useful metabolites into the culture broth have been investigated. These include pH cycling (Renaudin 1981), use of permeabilizing agents (Brodelius and Nilsson 1981), high ionic strength (Tanaka *et al.* 1985) and electroporation (Brodelius 1988), etc. Although these methods do enhance product release, they are usually detrimental cell viability and therefore not suitable for long-term process.

To overcome these problems, we have been working on a novel production systems consisting of isolated protoplasts (Akimoto *et al.* 2000, Aoyagi *et al.* 1996, 1998B; Tanaka *et al.* 1996B, 2000). In general, protoplasts have not been used for useful metabolite production but as an important material for cell fusion and genetic transformation. An advantage of using protoplasts is that the products are released freely into the broth with the double consequences of increasing overall productivity and facilitating down stream processing.

Furthermore, since many high molecular weight substances which are otherwise not accessible to the cells (due to the presence of cell wall) can directly access the cell membrane in protoplasts, the composition and concentrations of the metabolites produced by protoplasts may vary from those produced by cells. However, protoplasts are very fragile and can not be used for long term production process, and active protoplasts easily regenerate their cell walls during cultivation.

In order to solve these problems we used various gels as immobilization carrier (providing stability to the protoplasts) and used inhibitor of cell wall synthesis. Thus, active protoplasts were maintained for a long time without cell wall regeneration. We also proposed that immobilizing protoplasts is akin to providing them with an artificial cell wall. This concept has merits in that various useful functions can be freely incorporated in protoplasts by using artificial cell walls with such functions.

In this paper, we introduce about the high-speed production system of useful metabolites by protoplasts with artificial cell walls. This novel system solved the problems of conventional production systems using cells.

Key words : protoplast, artificial cell wall, alginate, elicitor, useful metabolite production

Materials and Methods

Plant cells

Wasabia japonica cells used for all the experiments secrete chitinase into the culture broth. Cell suspension culture were maintained and subcultured every 14 days in Murashige-Skoog medium (Murashige and Skoog 1963) supplemrnted with glucose (30 g/l) and 2,4-dichlorophenoxy acetic acid. (0.1 mg/l).

Results and Discussions

The elucidation of alginate as an elicitor

Promotion of the production of useful metabolites has been observed with some plant cells that are immobilized in alginate gel (Brodelius *et al.* 1979; Aoyagi *et al.* 1998B and references therein). In these caeses, we consider that the effect of alginate in promoting productivity is due to not only physical effects of immobilization but also to physiological effect. We have also observed that in the immobilized *W. japonica* cell culture, the specific chitinase productivity was much higher than that observed in suspension culture (Aoyagi *et al.* 1996; Tanaka *et al.* 1996A).

We studied about the effect of alginatre on the physiological activities of *W. japonica* cells. When alginate was added to *W. japonica* cell culture, cell growth was slightly inhibited but both the chitinase production and the specific chitinase productivity increased. On the basis of various investigations using various cultured plant cells, we concluded that alginate act as a kind of elicitor (Akimoto *et al.*1999; Aoyagi *et al.* 1996, 1998A, 2002).

Promotion effect of alginate on chitinase production was more remarkable when low molecular weight alginate (oligomer) was used (Aoyagi *et al.* 1996; Akimoto *et al.* 1999). Generally, the receptor for elicitor is located in the cell membrane, so we considered that some very low molecular weight alginates could pass through cell wall and access the receptor located in cell membrane. Furthermore, we considered that exposure of the cell membrane by removing cell walls would enhance the degree of elicitation. In comparison with free cells, addition of alginate to *W. japonica* protoplast culture result to 3 times increase in the chitinase productivity. We came up with the idea that we used alginate gel (artificial cell wall) as both elicitor and immobilization carrier (providing stability to the protoplasts).

Efficient production of chitinase by *W. japonica* protoplasts with artificial cell wall

We investigated about their efficient production of chitinase by *W. japonica* protoplasts with artificial cell wall [alginate gel] (Tanaka *et al.* 1996B). Protoplasts with artificial cell wall (immobilized protoplasts) could be cultivated in shake culture at low osmotic pressure without disruption. The chitinase productivity by the immobilized protoplasts (2.0 U/ml at 5 days) was much higher than that of the immobilized cells (0.36 U/ ml at 5 days). On the third day of cultivation, cell wall regeneration in the immobilized protoplasts was detected under light microscope. This implies that prevention of cell wall regeneration is pre-requisite for long-term process with protoplasts. When inhibitor of cell wall synthesis was added to the broth, active protoplasts were maintained for a long time without cell wall regeneration.

At high chitinase concentrations, product inhibition was observed. In order to prevent the product inhibition during the process, a system for continuous production with simultaneously recovery of chitinase was developed. Commercially available cheap chitin could be used to adsorb the produced chitinase (Yamamoto *et al.* 1995). A production column containing immobilized *W. japonica* protoplasts was coupled to a chitin column and by circulating the fermentation broth between the two columns, continuous production with simultaneous recovery of chitinase successfully carried out. The system was stable for a very long time and the chitinase productivity in this system was about 30 times higher than that obtained with suspended *W. japonica* cell culture system [Table 1] (Tanaka *et al.* 1996B).

TABLE 1. Comparison of chitinase productivity by free cells and immobilized protoplasts.

Type of cultivation	Cultivation time (day)	Total activity (U)	Production rate ([U/ml]/ days)
Suspension culture			
Free cell without chitin column	0-16	540	0.13
Immobilized protoplasts			
Batch without chitin column	0-8	864	0.60
Fed-batch with chitin column	0-25	26,000	3.00

Other studies about the application of this system

We have studied about the production of indole alkaloids (ajmalicine, catharanthine and tryptamine), which are synthesized through many enzyme reaction steps, using *Catharanthus roseus* protoplasts immobilized in guluronic acid rich alginate gels (artificial cell wall having a kind of elicitor function; Aoyagi *et al.* 1998B). The specific productivities of indole alkaloids by the protoplasts were much higher than those of the immobilized cells.

In the case of *Saccharomyces cerevisiae* cells, invertase was accumulated between cell membrane and cell wall. When *S. cerevisiae* protoplasts were immobilized in strontium alginate gel (having an excellent diffusion characteristics of invertase), invertase (molecular weight=270, 000) was secreted into the broth (Tanaka *et al.* 2000). The immobilized *S. serevisiae* protoplasts were used for invertase production in a bubble column reactor, and a high and stable level of invertase was maintained for 72 hours.

Conclusion

We have developed a system for high-speed production of useful metabolites

by plant and microbial protoplasts with artificial cell walls. The novel system has great potential in useful metabolite production. An advantage of the system is that the products are released freely into the broth with the double consequences of increasing overall productivity and facilitating down stream processing. Furthermore, since many high molecular weight substances which are otherwise not accessible to the cells (due to the presence of cell wall) can directly access the cell membrane in protoplasts, the composition and concentrations of the metabolites produced by protoplasts may vary from those produced by cells. This system solves the problems of conventional production system using cells.

Using this system, it may become possible to efficiently produce many useful metabolites which are usually difficult to produce using conventional methods.

References

Akimoto, C., Aoyagi, H. and Tanaka, H. (1999) Endogenous elicitor-like effects of alginate on physiological activities of plant cells. *Appl. Microbiol. Biotechnol.*, 52, 429-436.

Akimoto, C., Aoyagi, H., DiCosmo, F., and Tanaka, H. (2000) Synergistic effects of active oxygen species and alginate on chitinase production by *Wasabia japonica* cells and its application. *J. Biosci. Bioeng.* **89**, 131-137.

Aoyagi, H, Okada, M., Akimoto, C., Katsuyama, H., Yoshida, S., Kusakabe, I., and Tanaka, H. (1996) Promotion effect of alginate on chitinase production by *Wasabia japonica*. *Biotechnology Techniques,* **10**, 649-654.

Aoyagi, H., Yasuhira, J., and Tanaka, H. (1998A) Alginate promotes

production of various enzymes by *Catharanthus roseus* cells. *Plant Cell Rep.*, 17, 243-247

Aoyagi, H., Sakamoto, Y., Asada, M., and Tanaka, H. (1998B) Indole alkaloids production by *Catharanthus roseus* protoplasts with artificial cell walls containing of guluronic acid rich gel. *J. Ferment. Bioeng.*, 85, 306-311.

Aoyagi, H., Yasuhira, J., Kuno, A., Ono, K., Akimoto, C., Yoshida, S., and Tanaka, H. (2002) Distribution of alginate oligomers in *Catharanthus roseus* and *Wasabia japonica* cells cultured in a medium containing alginate oligomers. *Biotechnol. Lett.*, 24, 1125-1129.

Brodelius, P., Deus, B., Mosbach, L., Zenk, M.H. (1979) Immobilized plant cells for the production and transformation of natural product. *FEBS Lett.*, 103, 93-97.

Brodelius, P. and Nilsson, K. (1983) Permeabilization of plant cells resulting in release of intracellular stored products with preserved cell viability. *Eur. J. Appl. Microbiol. Biotechnol.*, 17, 275-280.

Brodelius, P. (1988) Permeabilization of plant cells for release of intracellularly stored products: viability studies. *Appl. Microbiol. Bbiotechnol.* 27, 561-566.

Murashige, T. and Skoog, F. (1963) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15, 473-479.

Tanaka, H., Hirao, C., Semba, H. Tozawa, Y., and Ohmomo, S. (1985) Release of intracellularly 5'-phosphodiesterase with preserved plant cell viability. *Biotechnol. Bioeng.*, 27, 890-893.

Tanaka, H., Kaneko, Y., Aoyagi, H., Yamamoto Y., and Fukunaga, Y. (1996A) Efficient production of chitinase by immobilized *Wasabia japonica* cells in double-layered gel fibers. *J. Ferment. Bioeng.*, 81, 220-225.

Tanaka, H., Yamashita, T., Aoyagi, H., Yamamoto Y., and Fukunaga,

Y. (1996B) Efficient production of chitinase by *Wasabia japonica* protoplasts immobilized in double-layered gel fibers. *J. Ferment. Bioeng.*, **81**, 394-399.

Tanaka, H., Kamogawa, T., Aoyagi, H., Kato, I., and Nakajima, R. (2000) Invertase production by Saccharomyces cerevisiae protoplasts immobilized in strontium alginate gel beads. *J. Biosci. Bioeng.*, **89**, 498-500.

Renaudin, J.P. (1981) Uptake and accumulation of an indole alkaloid, [14C] Tabernanthine by cell suspension cultures of *Catharanthus roseus* (L.) G. Don and *Acer pseudoplatanus* L. *Plant Sci. Lett.*, 22, 59-69.

Yamamoto, Y., Fukunaga, Y., Aoyagi, H., and Tanaka, H. (1995) Purification and characteristics of chitinase secreted by cultured *Wasabia japonica* cells. *J. Ferment. Bioeng.*, 80, 148-152.