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☘Prospects and Problems in the Large Scale Production of Metabolites from Plant Cell Tissue Cultures

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ABSTRACT

A wide range of plant products can be made directly by using plant cell tissue cultures. However, the economic production of even high-value products from such cultures has not been conclusively demonstrated. One problem is that rapid growth and high product yields often appear to be mutually exclusive with plant cell tissue cultures. Some level of cellular differentiation often is required for the expression of genes associated with product formation; only unorganized cells grow rapidly. Another problem results from the tendency of plant cells to form aggregates, which leads to a mixture of cell types in culture. The biological response is a function not only of the chemical environment but also the physical (e.g. hydrodynamic) environment which makes scale-up of suspension processes difficult. In addition, cell lysis due to high liquid shear is a significant design constraint. Some of these problems can be circumvented by using multi-stage continuous culture devices or with immobilized cell reactors. Emphasis will be on membrane entrapped cultures.

INTRODUCTION

The purpose of this paper is to provide an overview of the potential role of plant cell tissue culture in the commercial production of oils and fats. These products are of particular importance in the food and fragrance industries. Compounds of potential interest as oils or to the flavor and fragrance industry include jasmine, rose oil, chamomile, capsicum and cocoa butter. The emphasis in this paper is on the "factory" production of non-growth associated chemicals made by plants. The techniques described in this paper are applicable only to high value products.

Problems

The use of cell culture for chemical production has long been considered an attractive solution to the problems of extracting chemicals from the whole plant (1-4). The use of tissue culture insures a continuous supply of homogenous material which is independent of disease, weather, or politics; offers a significant opportunity to rapidly and greatly improve yields through selection of high-yielding variants, and can lead to the formation of novel products, primarily through the biotransformation of chemical analogs of natural substrates. The last feature is of particular interest in the pharmaceutical industry, but modified oils could prove to be of significant interest.

Although the "factory" production of chemicals from plant cells has been considered for 3 decades, it is only within the last year that a plant cell tissue culture process has been commercialized successfully. The Japanese corporation, Mitsui Petrochemical Industries Ltd., has begun producing shikonin from suspension cultures of *Lithospermum erythrorhizon.* (5) Shikonin can be used as a dye or pharmaceutical. Shikonin produced by Mitsui apparently will be used in lipstick.

Since the potential for the "factory" use of tissue cultures has been recognized for a long period and only one commercial process exists, it is clear that significant problems impede commercialization of tissue culture systems. Slow growth rates (doubling times are typically 20 to 100 h.) make experimentation tedious, require large bioreactors since the corresponding volumetric productivity is low, and result in systems very sensitive to contamination. Frustrating efforts further is the genetic instability often encountered with high yielding strains. The shear sensitivity of the cells constrains bioreactor selection. The major problem, however, is the low yields of non-growth associated products from most cultures. The latter problem can be attacked through selection procedures to isolate high-yielding variants and the selection of bioreactors which control the environment in a manner which leads to more complete gene expression. Both approaches are semi-empirical because of the paucity of knowledge about basic plant biochemistry. Nonetheless, some encouraging progress has been made on the selection of variants (6). Even with

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such variants, the problems of obtaining good gene expression remain and can be solved only by selecting the right type of bioreactor.

Bioreactors

The complication in the design of effective bioreactor systems is that high yields typically are associated with cells that are "partially differentiated" or in the form of organized tissues. Such cells usually have very low or zero rates of proliferation. Although a causal relationship is not established, most experience indicates that those conditions which enhance product formation suppress growth and vice versa. Thus, high yields usually can be achieved only in reactors separating growth and production functions (3). Two types of bioreactors can effectively separate these phases. For suspension cultures, multi-stage continuous cultures or batch analogs are possible. Alternatively, immobilized cell reactors can separate growth and production effectively. Interestingly, Mitsui uses a 2-stage batch process (9-day residence growth phase followed by a 14-day production phase) in the production of Shikonin. (5)

The scale-up of laboratory data to commercial size reactors is even more difficult for plant cell cultures than for most microbial fermentations. The primary difficulty is a result of cell aggregation caused by failure of cells to separate rather than by clumping. Plant cells are relatively large—often with diameters of 20 to 100 μm. Aggregates easily seen by the naked eye are common. When observed under the microscope, cells in the center of the aggregate are morphologically distinct from those on the edge. This alteration in morphology almost certainly represents a difference in biosynthetic capabilities. Cells on the edge of an aggregate have higher mitotic indexes than those in the center or free cells. Further, cells on the periphery respond to hormonal changes to form organized tissues while single cells do not. (7) The cells in the center have even been called "feeder" cells (8) to suggest that metabolites released by cells in the center of the aggregate are stimulatory to the cells on the edge.

Clearly the cells in the center of an aggregate experience a different environment from those on the edge due to the interplay of reaction and diffusion leading to concentration gradients in both substrates and products. (3) Further, a culture may be considered to consist of several cell types, essentially a mixed population even though the cells come from a single strain.

We speculate that the ratio of cell types will determine the biosynthetic capabilities of the culture. The ratio of cell types depends on the degree of aggregation which depends, at least in part, on the degree of physical mixing. Thus, reproducible results can be obtained in separate laboratories only if the degree of physical mixing is controlled as well as the normal parameters of medium composition, temperature, pH, inoculum preparation, etc. Since it is impossible to duplicate in a large vessel the same physical environment as in a small vessel, (9) the scale-up of plant cell suspension cultures may be very difficult. A number of examples are in the literature which show significant changes in growth rate (10), productivity in the formation of a secondary metabolite (11) and the pattern of product formation (12) due to changes in scale and physical mixing.

Another difficulty with large-scale systems is the shear sensitivity of the cells. Air-lift fermenters appear to be preferable when cell densities are less than 20 g dry weight/l and jar fermenters with modified paddle-type impellers for higher cell densities. (13) For suspension cultures these choices appear to be the best compromise between adequate mass transfer of nutrients (particularly O_2) and mixing and the reduction of cell lysis due to shear.

In addition to large scale systems it is important that the mixing in laboratory scale systems meet the same constraints for mass transfer and prevention of shear damage as well as providing a reproducible system for experiments. In our laboratory we have been working with "chemostat" type cultures. The use of chemostat theory requires "perfect mixing" in the culture vessel. We have adopted a modified culture vessel based on the design suggested by Wilson. (14) Aeration and agitation are provided by air sparging. An adequate approximation of the ideal reactor operation can be achieved by working at low Ca^{++} concentrations, with low density cell populations and at appropriate air flow rates. (15)

The use of such laboratory devices has been aimed at confirming the hypothesis that product yields can be enhanced greatly using suspension culture devices that separate growth from product formation. Both two-stage continuous flow systems (16,17) and fed batch systems (18) have shown dramatic yield improvements. Sahai and Shuler (17) have compared the productivity of tobacco cells for phenolics production in a two-stage system to that in a single-stage system with the same total residence time. The productivity in the two-stage system was 1.74 times higher for total phenolics and 3.38 times higher for extracellular phenolics than in the single-stage system. Chemostat operation allows cells to reach a balanced growth state and is ideal to study the intrinsic physiological state of a culture. With the two-stage system, inputs which might normally be growth inhibitory can be applied to the second stage without fear of cell washout due to the constant reinoculation of the second stage by the effluent from the first stage. The addition of glucose to the second stage of the above system led to an increased cell population and a decreased productivity. The system was glucose-limited; if it had been limited by another nutrient, the extra glucose carbon might well have been directed into phenolics synthesis. Phenylalanine is a potential precursor of phenolics metabolism but also can be toxic to cell growth. When a pulse of phenylalanine was added to the second stage, the growth decreased and phenolics production increased until sufficient phenylalanine had been metabolized or washed out to allow the system to return to the original steady-state. A multistage continuous culture system is well suited to basic studies on factors controlling plant cell biochemical response as well as offering a potentially attractive device for improving the yield of non-growth associated chemicals.

Nonetheless, suspension culture systems are difficult to operate for extended periods of time without mechanical or contamination problems. An alternative approach which may be particularly attractive for commercial scale production of plant chemicals such as oils is immobilized cell reactors.

The potential advantages of immobilized cell reactors are:

- Continuous operation at high flow rates without concern about cell washout
- Problems due to genetic instability can be reduced greatly since cell replication of the immobilized cells is greatly repressed
- Contamination is no greater problem for immobilized plant cell systems than for other immobilized systems such as bacteria. Thus the impact of the slow growth rates of the plant cells can be reduced.
- The cells are protected from liquid shear allowing the engineer to choose fluid mixing characteristics based solely on mass transfer characteristics.
- Cell aggregate size can be better controlled. Potentially the optimal mixture of cell types can be achieved if such a mixture is a physical reality. If an optimal aggregate

size exists, the system can be designed to ensure that this optimal aggregate size is uniformly achieved and maintained – something a suspension culture process cannot do.

- The system allows the separation of growth (prior to immobilization) and production. With the immobilized cell system the medium can be optimized solely for product formation. The suppression of all replication is desirable to prevent cell overgrowth and the disruption of the immobilizing matrix.
- Metabolic inhibitors which normally would accumulate in a batch suspension culture are "dialyzed" away and may allow the expression of metabolic pathways which normally would be suppressed.
- Because of the high cell densities achievable in immobilized systems and the potential to increase specific productivity (amount of product per unit weight of cells per unit time) because the mixture of cell types is controllable, then it should be possible to dramatically improve volumetric productivities (amount of product per unit volume of reactor per unit time) over batch reactors.

Although these advantages are important, entrapped cell cultures do have limitations. The primary one is that the product must be excreted into the extracellular medium. Most plant products are normally sequestered in vacuoles inside the plant cell. However, techniques for the release of normally intracellular compounds are being developed. Cyclic changes in pH can result in the cyclic release of vacuole-stored alkaloids. (19) Solvents, such as DMSO, can be used to obtain product release without irreversible damage to the culture (20,21). Further, there exists at least circumstantial evidence to indicate that the microenvironment about immobilized cells (perhaps due to the high cell density) can lead to excretion of normally intracellular compounds. Alferman et al. (22) give experimental data that are consistent with the hypothesis that vacuoles can become saturated and that continued product formation results in excretion.

Immobilized Cell Reactors-Choices

Immobilization techniques that can be applied to plant cells appear to be limited by the sensitivity of plant cells to chemicals (23) which normally are used to link cells covalently to solid surfaces or as a cross-linker in some entrapment schemes. Entrapment in gels of agar, agrose, alginate and carrageenan (20-23) or entrapment between or within membranes (3,24,25) have been used successfully with plant cells.

Gel entrapped systems are generally less expensive to initiate than membrane reactors. Further, gel systems usually have less severe problems with gas transfer. However, membrane reactors have a number of compensating advantages. Most gels appropriate to plant cell immobilization are very compressible. Large packed columns of such material would be difficult to operate due to large pressure drops and non-uniform flow patterns for the fluid. Membrane reactors offer much better control of the fluid dynamics and flow distribution and are, in principle, easier to scale-up from laboratory data. With gel systems part of the flow problem can be circumvented by using a fluidized bed operation, but bead abrasion then becomes a concern. In bead systems when the catalyst is exhausted fresh beads must be made. Many membrane devices are potentially reusable since old cells can be flushed out and fresh cells (of the same or of a different strain) can be pumped into the system.

Membrane Reactors

The authors' research group has concentrated on membrane reactors. Five possible configurations for membrane reactors are: hollow fiber units, flat plate systems, spiral wound units (essentially a flat plate system that has been wound in a spiral), tubular membrane reactors and multimembrane reactors. The characteristics of these reactors

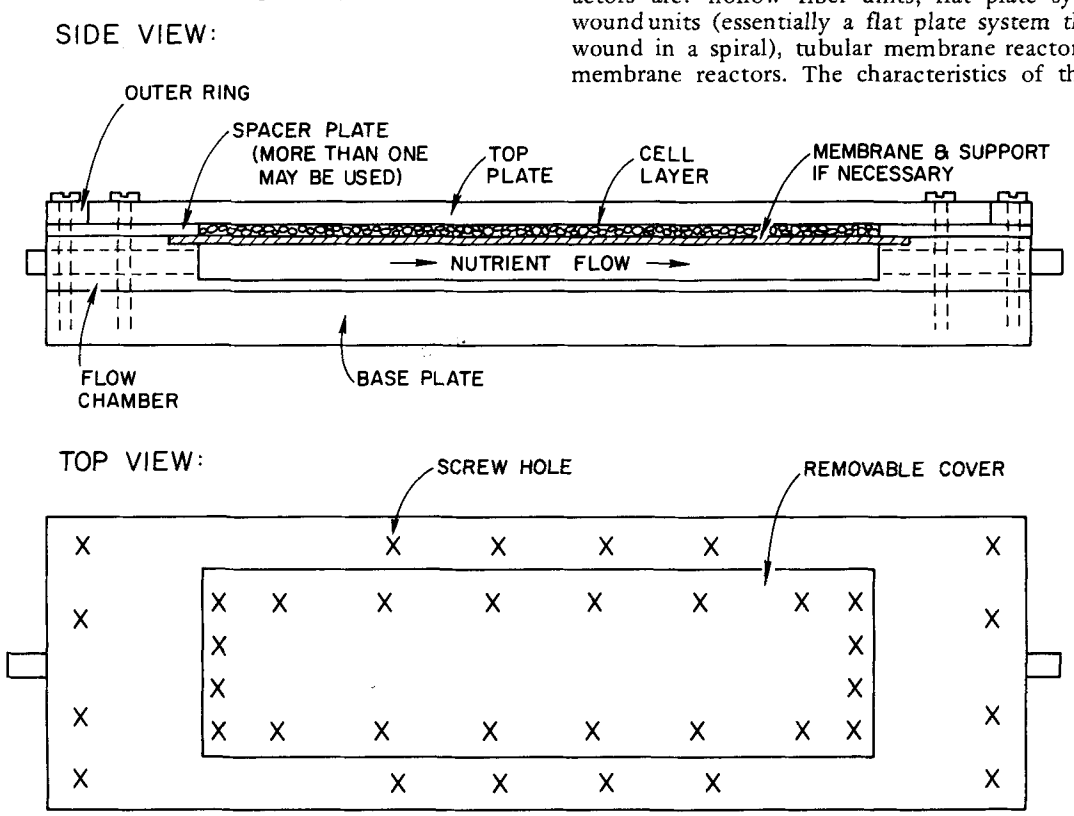

FIG. 1. One-sided flow reactor. The current design uses a 150 mesh stainless steel screen as a "membrane". Previous experiments have used a polypropylene sheet with 125 μm pores as a membrane and a Nucleopore membrane (0.4 and 0.6 μm pores) supported by a porous polypropylene sheet. From Ref. 27, with permission.

have been reviewed recently. (27) The specific methodology of using membrane reactors for plant cells also has been reviewed recently (28).

Consider a flat plate reactor as shown in Figure 1. Substrate enters the cell layer by diffusion or pressure driven flows. Substrate is consumed and converted to product. The product will then diffuse into the cell free stream. The flat plate system shown allows the direct sampling of the cell layer through a removable top plate. The depth of the cell layer can be altered by the investigator.

The system has been used for the production of extracellular phenolics from tobacco cultures. A typical experimental result is displayed in Figure 2 for a flat plate system with flow both over and under the cell layer. The reactor is part of a previously described system (24) in which effluent is recycled back to a recycle chamber. Fresh feed is continuously introduced into the chamber, and a product stream is withdrawn at an equal rate.

Eleven experiments similar to that described in Figure 2 have been accomplished. Preliminary results demonstrate that product excretion can be maintained for at least 110 days. The productivity on a dry weight basis is ca. 20 μg phenolics/g cells-h, which is higher than can be achieved in a two-stage continuous culture system (12 μg/g-h) or in batch culture (ca. 4 to 7 μg/g-h). This higher productivity coupled with the increased cell concentration and continuous operation would lead to about a 20-fold advantage in volumetric productivity for the immobilized system over the batch system.

These preliminary experiments also have demonstrated that the membrane resistance to mass transfer is important. Initial experiments were performed with Nucleopore membranes with 0.4 to 0.6 μm pore sizes over a membrane support. After about five weeks of operation the system was dismantled. Extracellular phenolics production had been low. The total volume occupied by the cells had decreased, the cells were very elongated, the cell mass was dark brown in color and the cell layer had a gelatinous cohesive character. Scattered throughout the portion of the layer nearest the membrane were small, hard lumps of lighter colored tissue (ca. 1 to 2 mm in diameter). Microscope observations of this tissue indicated a higher level of metabolic activity.

When the Nucleopore membrane was removed and the membrane support (125 μm pores) alone was used to retain the cells, the cellular response was altered significantly. The 125 μm pores allowed complete retention of the cells and improved mass transfer rates (about a 10-fold increase in oxygen permeability and a 50-fold increase in the transfer of the artificial plant hormone 2,4D and related compounds with similar molecular weights and configuration). With the large pore size membrane the cells were relatively unchanged in color and morphology after 2500 hr of operation. The cells had high levels of peroxidase activity and improved specific productivities for extracellular phenolics. No organized structures were observed in these experiments using an LS medium with reduced phosphate (30% of normal).

However, when the artificial auxin 2,4-D was removed from the medium, hard nodules of whitish tissue with high respiratory activity were formed. These nodules are primitive forms of cellular organization. Although the removal of auxin reduced extracellular phenolics productivity by 50%, the formation of other potential products was almost certainly induced. For products which come solely from organized tissues (e.g. cocoa butter), the inducement of cellular organization will likely be a prerequisite to obtaining high product yields.

The phenolics appear to inhibit their own formation. When the feed flow rate was decreased by 50%, there was little change in extracellular phenolics concentration as long as the flow rate was between 1.0 and 10 ml/hr. This observed insensitivity to flow is consistent with a hypothesis of phenolics toxicity. The in situ removal of phenolics could improve productivity.

Alternate Approaches

Thus far this paper has focused on the possibility of using

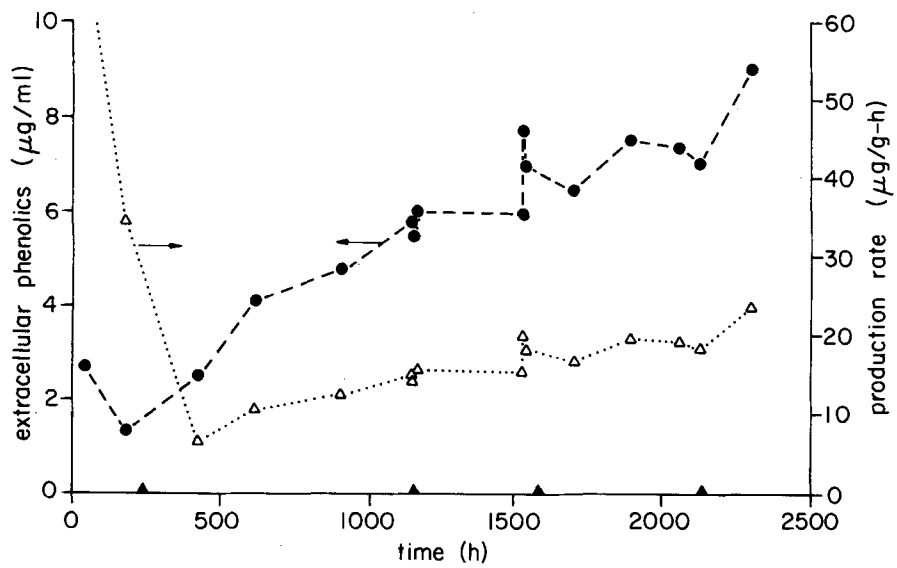

FIG. 2. Results of a typical experiment with the 2-sided flow reactor. An LS medium with 2,4D as the auxin was used. The depth of the tobacco cell layer was 3.2 mm. A polypropylene 125 μm membrane was used. At 240 hr after start-up the feed flow rate was changed from 10.2 ml/hr to 1.04 ml/hr. At 1152 hr 20 ml of conditioned and filtered sterilized fluid from a culture contaminated with an unknown bacterium was added; at 1524 hr an additional 20 ml was added. The presence of the bacteria had dramatically improved phenolics production in a contaminated culture. These additions tested whether an extracellular compound produced by the bacteria could induce higher levels of phenolics production. The results were inconclusive. At 2126 hr a power failure for 3 hr decreased oxygen availability.

tissue cultures for chemicals production. However, some oils and chemicals are made only in rather specialized organs. The use of organ cultures for chemicals production has been explored little. Such cultures would offer difficult challenges in bio-reactor design. Nonetheless, it may be quite valuable to consider such systems for products from such tissues.

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Production of Protein and Fatty Acids in the Anaerobic Fermentation of Molasses by *E. ruminantium*

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ABSTRACT

Production of protein and volatile fatty acids by anaerobic digestion of blackstrap molasses was investigated. This protein may have potential as a feed for animals (and, we hope, for humans) in the near future. Volatile fatty acids can be further fermented to produce methane. Fermentation of molasses by *Eubacterium ruminantium* was studied in a chemostat at a constant temperature of 37 C. This study focused on kinetics of growth of the pure culture. The maximum rate of protein production of about 0.326 g/l/hr was obtained when the pH and retention times were 6.2 and between 5 and 7 hrs, respectively. Average cell yield was 12.6% and carbohydrate conversion was 82 to 99%. Volatile fatty acids also were produced, with acetic acid and n-butyric acid being the predominant products. Two different kinetic models were used to fit the experimental data. The kinetic parameters obtained for the Monod model were: $\mu max = 0.207$ (1/hr); $k_s = 0.165$ g/l.

INTRODUCTION

A need exists for developing protein, preferably from materials not directly consumed by animals or humans (1). If a usable protein can be produced by fermentation of carbohydrate-rich agricultural wastes, it might be substituted for soybean meal. Benefits would be an economic solution to waste disposal problems; use of waste as substrate which has greater negative cost value; a feed supplement rich in crude protein for animal feed; a possible protein which can be purified for human consumption; the independence of protein production on agricultural land, and energy production in the form of methane.

In Germany during World War I bakers' yeast, *Saccharomyces cerevisiae,* was grown with molasses and ammonium salt for consumption as a protein supplement. Protein is produced by the aerobic cultivation of lactose fermenting yeast, *Saccharomyces fragilis* on cheese whey (2,3). The 'Symba' Process, using an *Endomycopsis* to hydrolyze starch in wastes followed by Torula yeast grown on sugar, was developed in Sweden many years ago (4). Callihan (5) et al. used *Cellulomonas uda* to ferment bagasse for protein production. Gautreaux (6) fermented sweet potato waste using primary sludge from a sewage treatment plant for producing protein and methane. An advanced commercial process for protein production from methanol has been developed by Imperial Chemical Industries, Ltd. They started up a 60,000 metric ton/year plant in 1981 at Billingham, England, and now are trying to adapt the technology to use sugar and other substrates (7). Besides ICI and Phillips Petroleum, a number of other organizations like Germany's Hoeclist and Japan's Mitsubishi are investigating SCP production for human consumption.

Most of the above processes use aerobic fermentation. Though the technology of aerobic fermentation is well developed, in this research anaerobic fermentation was used. This is because of the following proven advantages of anaerobic fermentation and the limitations of aerobic fermentation:

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