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Patents and Literature Review

Bioprocessing Technology for Plant Cell Suspension Cultures

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ABSTRACT

Considering various forms of in vitro plant tissue cultures, cell suspension culture is most amenable to large-scale production of natural compounds, owing primarily to its superior culture homogeneity. This fact has already been demonstrated in several large-scale applications, including the commercial shikonin process. The scope of this work is to review the state of the art in bioprocessing technologies pertinent to the secondary metabolite production from suspension cultures of callus cells. In the first part of the review, plant cell physiology relevant to bioprocess design is considered. This is followed by an in-depth discussion on the bioreactor design and operation and its effect on plant cell suspension cultures. Finally, recent commercial exploitation and development are summarized. Following the review, related patents and literature are listed.

Index Entries: Bioprocessing technology; plant; suspension culture; callus; secondary metabolite; bioreactor.

INTRODUCTION

Plants are important sources of many useful compounds. New technologies to obtain these compounds, usually secondary metabolites, in large quantities have been proposed through the use of in vitro tissue culture techniques (*1,2*). Two forms of tissue cultures have received most attention in the production of plant metabolites. They are organ cultures and dedifferentiated callus cultures. Organ cultures, especially hairy root

cultures, offer an attractive means for the synthesis of secondary metabolites that are associated with cellular differentiation. This form of tissue culture, however, is difficult to scale up. On the other hand, certain secondary metabolites may not accumulate to any appreciable level in dedifferentiated callus cells because of the lack of cellular differentiation. Suspension cultures of callus cells, nevertheless, possess a superior culture homogeneity compared to organ cultures of roots or shoots, and therefore are more amenable to large-scale production of natural compounds. Many of the fermentation technologies developed for submerged microbial cultures can be applied in the plant suspension culture systems. Technological feasibility of large-scale suspension plant cell cultures has already been demonstrated in Japan during the past decade in the shikonin and berberine production by Mitsui Petrochemical Industry Ltd. and ginseng production by Nitto Denko Co. (*2,3*).

The scope of this review is to focus on recent advances in bioprocessing technologies and commercial developments pertinent to suspension cultures of dedifferentiated callus cells for secondary metabolite production. Reviews on organ cultures and immobilized cell cultures can be found elsewhere (*4,5*). Since most products of interest from plant cell culture are low molecular weight compounds such as alkaloids, rather than macromolecules like proteins, downstream separation usually does not pose a serious problem. Therefore, only upstream processing will be discussed in this review.

In the first part of the review, plant cell physiology relevant to bioprocess design is considered. This is followed by an in depth discussion on the bioreactor design and operation and its effect on plant cell suspension cultures. Finally, recent commercial exploitation and development are summarized.

PLANT CELL PHYSIOLOGY

Synthesis of secondary metabolites is strongly related to the cell physiological state, which is, in turn, affected by the regulation of the culture environment or by genetic selection and manipulation (*6–8*). Physiological considerations of secondary metabolite production is discussed below, with emphasis given to the effects of macronutrients, cell division and differentiation, elicitation, metabolite transport and storage, and the implication of these effects on the development of culture strategies for large-scale plant cell suspensions.

Macronutrients

Sugar catabolism by plant cells is believed to be similar to catabolism used by other heterotrophic cells (*5,9*). Among several carbohydrates, sucrose tends to be the most efficacious carbon and energy source for

cultured plant cells, especially with respect to secondary metabolite production, although precisely how the effect is mediated is unclear (*5*). Sucrose introduced externally to plant cell suspensions is usually rapidly hydrolyzed to glucose and fructose, which are then taken up by a passive or active transport process, depending on the species (*10,11*). This hydrolysis appears to be catalyzed by a wall-bound or extracellular acid invertase (*11*). After hydrolysis, fructose is generally utilized only after most of the glucose has been consumed, e.g., in carrot cells (*12*). However, no preferential utilization was observed in cultured rice cells (*10*). In *Anchusa officinalis* cells that produce rosmarinic acid, glucose was only slightly preferred over fructose in batch cultures, yet glucose was taken up by the cells much faster than fructose in perfused cultures (*13*). Cresswell and coworkers (*9*) found higher β-glucosidase activities in cell suspension culture of *Solamum tuberosum* using 2% fructose compared to sucrose at the same concentration. Glycosylated secondary products, which are more easily accumulated in the vacuole and less cytotoxic than aglycones, can be hydrolyzed by β-glucosidase (*9*). A reduction in the potential of cells to store secondary products as glycosides is likely to result in a parallel reduction in the potential for secondary product accumulation if the products are cytotoxic in their aglycone form (*9*). This speculation, however, is challenged by numerous demonstrations of spatial separation of glycosides from the degradative enzymes (*14*).

In addition to serving as carbon and energy sources, sugars also affect the osmotic pressure of the medium. Improved secondary metabolite production has been observed under osmotic stress by adding high concentrations of sucrose or sugar alcohols that are not metabolized by plant cells, such as mannitol, sorbitol, or xylitol (*15,16*). The osmotic environment also has an impact on cell morphology, which in turn can affect the culture viscosity (*13,17*).

Nitrogen is supplied to most plant cells as a combination of ammonia and nitrate salts. The ratio of these salts is important in most cases. Ammonia is usually taken up by the cells in the early stages of the culture leading to a decrease in pH, followed by an increase during assimilation of nitrate (*5,18*). This pH alteration generally does not cause adverse effects on cell growth or secondary metabolite production, and therefore the addition of buffering agents to the medium is not necessary (*19*). Ammonia assimilation involves a glutamine synthetase–glutamate synthase (GS-GOGAT) system (*5*). Once ammonia is assimilated into glutamine, the nitrogen groups can be transferred to other carbon skeletons via transamination reactions (*20*). These reactions provide the amine groups for other amino acids (*20*). During nitrate assimilation, nitrate is first reduced to ammonia by nitrate reductase and nitrite reductase (*5*).

The role of nitrogen source in the secondary metabolism is less clear, although the nitrogen source has shown a significant impact on secondary metabolite synthesis in many plant cell culture systems, including the production of shikonin by *Lithospermum erythrorhizon* (*21*). During the

synthesis of anthocyanins by grape cells, reduction of nitrate in the medium to a critical level resulted in a switchlike enhancement of anthocyanin production. It was speculated that a nitrate-sensitive tonoplast ATPase might be involved in the uptake of anthocyanin into the grape cell vacuole. By avoiding nitrate inhibition, anthocyanin uptake into the vacuole was enhanced, which, in turn, led to the increased anthocyanin production (*22*).

Phosphorus is supplied in the form of inorganic phosphate in plant cell cultures and is usually taken up by cells rapidly and stored in the vacuole, mainly as orthophosphate (*23*). Growth under phosphate limitation, therefore, usually does not follow the Monod model, which is based on the availability of substrate in the medium (*4,13*). Phosphate is required for the biosynthesis of nucleic acids, phospholipids, nucleotides, and sugar phosphates (*24*). It is also important in cell energetics through its effect on ATP synthesis (*25*). There have been many reports on improved secondary metabolite synthesis on phosphate limitation. For example, Schiel and coworkers (*26*) maintained the intracellular phosphate concentration in *Nicotiana tabacum* cell suspensions at low levels by means of a fed-batch technique and obtained a significant improvement on the production of cinnamoyl putrescines. In *A. officinalis* cultures, however, rosmarinic acid production was not affected by high levels of phosphate (*18*). It is difficult at this point to reach any unequivocal conclusion for predicting the culture's response to phosphate limitation. It is generally believed, however, that for certain culture systems where cell growth is closely correlated with secondary metabolite production, the effect of phosphate may come from its role in regulating the cell growth rate (*5,27*).

Cell Division vs Differentiation

Lack of cellular differentiation is often considered as one of the major limitations in utilizing dedifferentiated callus cells for secondary metabolite production. Although plant cells are totipotent and hence should carry the genes for secondary metabolites as in the whole plant, these genes may not be expressed in a dedifferentiated state (*28*). Cellular differentiation in cultured callus cells can be manipulated in part by the use of exogenous growth regulators, such as auxins, cytokinins, gibberellins, abscisic acid, and ethylene (*6*). An increase in the auxin level often leads to dedifferentiation via stimulation of cell division and in many cases diminishes the level of secondary metabolites (*6*). For those secondary metabolites present in specific tissues that require developmental control, such as anthocyanins, there could be a strong inverse correlation between cell growth and differentiation. For example, anthocyanin accumulation in *Vitis vinifera* suspension cultures increased only in the stationary phase when cell division ceased (*27*). Cell division can be reduced using low phosphate media or addition of DNA synthesis inhibitors, such as aphidicolin (*27*). Caution should be taken, however, when

using an inhibitor of protein synthesis to retard cell division. For example, addition of cycloheximide inhibits both cell division and anthocyanin synthesis in *Vitis* cultures (*27*). In this case, cycloheximide may inhibit the synthesis of the key enzymes in anthocyanin synthesis. In carrot suspension cultures, low 2,4-D concentration induces both anthocyanin synthesis and embryogenesis (*29*). Induction of chalcone synthase (CHS) activity by removing 2,4-D from the medium closely correlates with the induction of anthocyanin synthesis, and peaks of anthocyanin production rate and CHS activity coincide with each other (*29*).

For those secondary metabolites that are not localized in specific tissues, differentiation may not be critical in the metabolite synthesis. For instance, accumulation of betacyanin is observed in all tissues in roots of red beet, and cellular differentiation is not essential for the production of this pigment in cell cultures (*27*).

Transport, Storage, and Turnover of Secondary Metabolites

Both active and passive transport have been suggested for the translocation of secondary metabolites in cultured plant cells (*30,31*). During active transport, proton gradients are generated by ATPases. Plant cells have at least two types of electrogenic H^+-translocating ATPases. A vanadate-sensitive ATPase is enriched in the plasma membrane and a vanadate-insensitive, anion-sensitive ATPase is enriched in the tonoplast (*30*). Active transport is also involved in the excretion of some secondary metabolites, such as the secretion of berberine by *Thalictrum minus*, in which ATPase inhibitors block the alkaloid efflux (*32*). Specific carrier proteins, utilizing the energy of the pH gradient across the membrane, may be involved in the active transport. Carrier-mediated transport has been observed for alkaloids, acylated anthocyanins, and coumaryl glucosides such as esculin (*31*). Wink (*28*) suggested that reduced expression of carrier proteins was partly responsible for the low production of quinolizidine alkaloids in *Lupinus polyphyllus* cell cultures.

Passive diffusion has been proposed as a conceivable mechanism for the translocation of lipophilic secondary compounds across membrane (*31*). Lipophilic metabolites such as the aglycone esculetin and weakly basic alkaloids such as ajmalicine (pKa = 6.3) have a higher potential for diffusion than polar and charged molecules like lupanine and S-reticuline (both basic alkaloids with pKa > 9) (*31*). Passive diffusion is usually coupled with ion-trapping, which is responsible for the accumulation of lipophilic alkaloids in vacuoles (*30*). Uncharged lipophilic alkaloids diffuse into the acidic vacuoles where they are protonated and trapped as cations to which tonoplast is only slightly permeable or completely impermeable (*33*).

It has been shown that the initial uptake of nicotine by *A. pseudoplatanus* is increased with a higher extracellular pH (*31*). Therefore, the efflux of secondary metabolites may be enhanced by reducing the medium pH or

increasing the cytoplasmic pH. Increasing the internal pH by treating the cells with NH_4^+ leads to a rapid release of endogenous alkaloids (*34*), whereas acidifying proved effective for the uptake of exogenous alkaloids (*35*). However, this may not be sufficient in all cases: In *C. reseus* suspensions, secretion of ajmalicine could not be induced after acidification of the medium by 0.6 pH units, although lipophilic probes (accumulated in vacuoles by cation-trapping) were released upon this treatment (*36*). In this system, ajmalicine was mostly accumulated in a form that was strongly retained in the vacuoles and not directly available for diffusional processes (*36*).

Vesicle-mediated synthesis and transport of secondary metabolites may also be important as a mechanism controlling efflux and vacuole loading (*33*). These membrane-bound vesicles derive mainly from specialized regions of endoplasmic reticula and are closely associated with Golgi stacks (*33*). In cell cultures of *L. erythrorhizon*, shikonin accumulated in the vesicles derived from electron dense, spherical bodies formed in highly elongated, rough endoplasmic reticulum. Most of these vesicles appeared to fuse with the plasma membrane to deposit the contents, consisting mainly of shikonin granules, lipids, and proteins, on the outside of the cell wall (*37*).

Most secondary metabolites are synthesized in the cytosol and then translocated into a storage compartment, in many cases, stored as glycosides (*9*). If a suitable storage compartment is not available, secondary metabolites may be subject to rapid turnover. For example, in the production of quinolizidine alkaloids by *Lupinus polyphyllus* cell cultures, large amounts of alkaloids were secreted into the medium, but quickly disappeared (*28*). This phenomenon may be owing to the presence of oxidative enzymes, such as peroxidases, in the medium, which leads to product degradation (*28,33,38*). Inhibition of extracellular peroxidase activity by the addition of Mn^{2+} led to high levels of excreted pterocarpans in chickpea suspension cultures (*38*).

Elicitation

Elicitor-induced accumulation of secondary metabolites has received much attention during the past decade. According to Eilert (*39*), there are four major groups of elicitors triggered by various types of plant-microbe interactions:

1. Compounds released by microbes that can be recognized by plant cells through specific membrane receptors.
2. Plant cell wall components released by the action of microbial enzymes.
3. Cell wall components from the microorganisms released by plant enzymes.
4. Endogenous compounds, formed and released by plant cells in response to various stimuli, such as a pathogen attack.

DiCosmo and Misawa (*40*) suggested the possible involvement of a second messenger in the process of elicitation. This compound would transmit signals from the plasma membrane and trigger transcription and translation of proteins. Calcium ions and ethylene have been suggested as possible second messengers (*41,42*). Recently, a mathematical model was developed to analyze the regulation and dynamics of elicitation involving a second messenger (*43*).

Considering the broad range of metabolites, both phytoalexins and nonphytoalexins, found in cell cultures in response to elicitation, generalization of the action of elicitors is very difficult. Optimization of the elicitation process hence can only be achieved empirically. Nevertheless, several factors are known to affect the elicitor-induced accumulation of secondary metabolites, which may serve as guidelines in optimizing culture elicitation. These factors include elicitor specificity and concentration, period of elicitor contact, cell line variations, time course of elicitation, growth stage of cell culture, growth regulators, as well as culture condition and nutrient composition (*39,44*). Eilert (*39*) extensively surveyed data in the literature and concluded that treatment of a particular cell culture with different elicitors would result in the accumulation of the same compound, if the culture would respond at all. The required elicitor concentration may depend on the type of elicitors. Pure elicitor compounds may be active in trace amounts, whereas concentrations up to 5% are required for optimal activity of complex fungal homogenates. The elicitor dose-response curves usually show a sharp optimum. Accumulation of secondary compounds starts within hours after elicitor treatment. The accumulation is generally transient, and is subject to a fast turnover. Most cultures showed response to elicitation only in the growth phase, during which secondary metabolism is not active. Treatment with additional stimuli at a later stage is usually not fruitful, since secondary metabolite accumulation has already been induced.

BIOREACTOR DESIGN AND OPERATION

Morphologically, plant cell suspensions are somewhat similar to microbial cultures, and therefore the design of plant cell bioreactors largely has been based on the reactor technology applied in submerged microbial fermentation. However, there are several distinct differences between microbial cell suspensions and the suspension cultures of callus cells, particularly at high cell densities. These differences must be taken into consideration in plant cell bioreactor design. Bioreactors for plant cell and organ cultures have been reviewed by several investigators (*4,5,45–48*). In this section, emphasis is placed on reactors for suspension cultures at high cell densities. Examples of bioreactors for plant cell suspension cultures are summarized in Table 1 and schematic representation of these reactors is shown in Fig. 1.

Table 1
Bioreactors for Plant Cell Suspension Cultures (*see* Fig. 1)

Reactor	Reference
1. Stirred tank reactor with bubble aeration	
1.1 Flat turbine	*75,78,85,116*
1.2 Gate paddle with a spiral sparger	*77*
1.3 Cell-lift impeller	*113*
1.4 Large paddle	*78*
1.5 Pitched blade turbine	*13*
1.6 Helical stirrer	*75,76*
1.7 Vibromixer	*83*
1.8 Marine propeller	*13,83*
1.9 Anchor impeller	*83*
1.10 Hollow paddle	*79,85*
2. Stirred tank reactor with bubble-free aeration	
2.1 Helical ribbon impeller with surface aeration & surface baffles	*74*
2.2 Membrane stirrer	*64*
2.3 Flat turbine with coiled membrane aeration tubing	*13,95,96*
3. Rotating drum reactor (RDR)	
3.1 RDR with surface aeration and baffle plate	*86*
3.2 RDR with bubble aeration	*84*
4. Column reactors	
4.1 Internal-loop air-lift with axial-flow impeller	*83*
4.2 Bubble column	*57,80*
4.3 Internal-loop air-lift with aeration into the annulus	*4*
4.4 Internal-loop air-lift with aeration into the draft tube	*55*
4.5 External-loop air-lift	*99*
5. Perfusion reactors	
5.1 Filtration stirred tank bioreactor	*13,95,96*
5.2 Stirred reactor with a sedimentation column	*113,117*
5.3 Spin-filter reactor	*127*
5.4 Filtration bubble column	*125*

Rheological Characteristics

Most plant cell cultures are viscous at high concentrations. This in general is a result of the high solid content in the dense culture (packed cell volume, PCV, can be as high as 80% in some cases, depending on the cell water content [*49*]), whereas the spent culture media are usually low in viscosity (*49–53*). Plant cell suspension cultures behave as non-Newtonian fluids (*51,54*). Power-law models including Bingham plastics (*53*), pseudoplastics (*51,52*), and Casson fluids (*55*) have been used to describe the rheological characteristics of plant cell suspensions. In power-law rheological models, shear stress (τ) and shear rate (γ) are correlated as follows (*54*):

$$\tau = \tau_0 + K\gamma^n \quad (1)$$

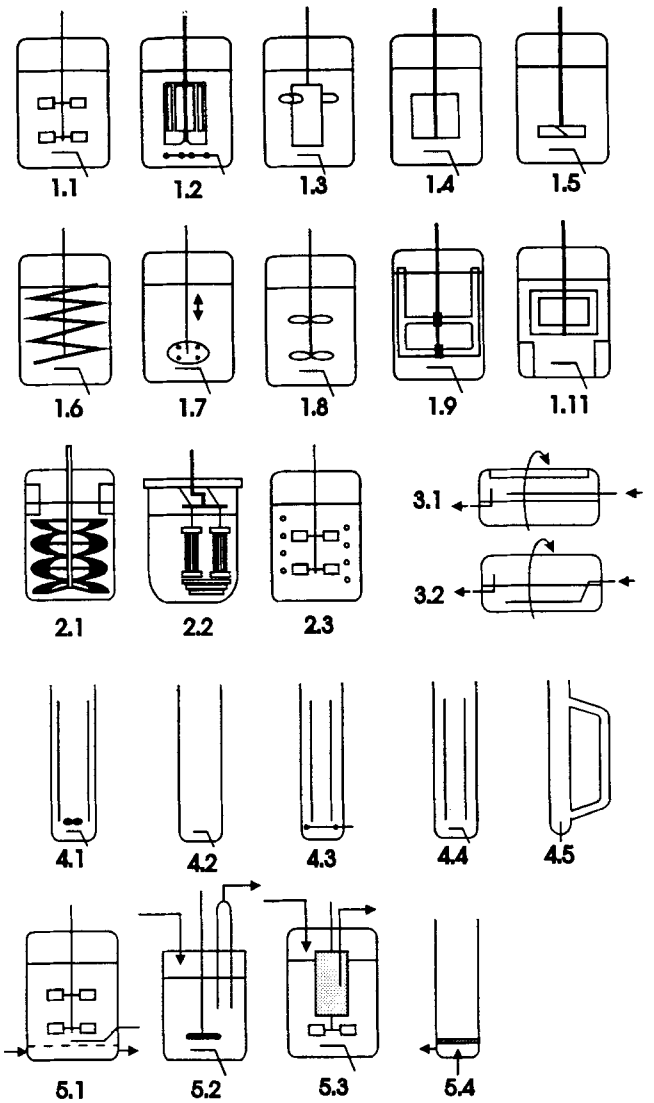

Fig. 1. Plant cell bioreactors as of Table 1.

where K = consistency index, τ_o = yield stress, n = flow behavior index. For Bingham plastics, $n = 1$ and $\tau_o \neq 0$. For pseudoplastic fluids, $n < 1$ and $\tau_o = 0$. For Casson fluids, $\tau^{1/2} = \tau_o{}^{1/2} + K_c \cdot \gamma^{1/2}$, where K_c is the Casson viscosity. In plant cell cultures, the consistency index is greatly affected by aggregate size and cell concentration, whereas the flow behavior index is not (*4,53*). Culture rheological properties can affect mixing and oxygen transport substantially. For instance, in pseudoplastic fluids, apparent viscosity is lower under higher shear. In this case, mixing and bubble dispersion will be better in the impeller region where high shear exists, and the area away from the impeller will experience a higher apparent viscosity and may lead to poor mixing and oxygen transfer.

It is very critical to choose a suitable method for the measurement of rheological properties of plant cell suspensions, because of the presence of cell aggregates. Rotating cylinder viscometers have been used most often for plant cell suspensions. They are, however, not altogether suitable for particulate suspensions, as they suffer from phase separation in the region of the bob, gravity settling of particles causing a nonhomogeneous suspension, and even the destruction of cell aggregates (*4,51*). These problems can be partially alleviated using a suitable impeller system including turbine impeller, helical ribbon impeller and anchor impeller (*51*). The major drawback of these systems is the limited operating range of shear rates. Using a double-gap rotating cylinder viscometer, time-dependent thixotropic behavior and yield stress were observed in *C. roseus* cell cultures with large aggregates, at a cell concentration of 100 g fresh wt/L (*51*). When an anchor impeller was used, however, there was no evidence for either the thixotropic behavior or the yield stress. In addition, *C. roseus* cell cultures, with only small aggregates, failed to exhibit a yield stress or thixotropic behavior with the double-gap rotating cylinder viscometer. Apparently, progressive breakup of large cell aggregates during measurement using the rotating cylinder viscometer led to the observation of thixotropic behavior and yield stress (*51*).

As pointed out in the preceding section, culture rheological characteristics can be altered by changes in the medium osmolarity. In typical batch plant cell cultures using sucrose as the carbon source, medium osmolarity initally increases (owing to hydrolysis of sucrose), then gradually decreases and approaches zero toward the stationary phase, resulting from the depletion of nutrient components (especially the sugars) from the culture medium (*13,17*). Under such a low osmolarity, cell enlargement may occur. Although large cells may possess a larger storage compartment, they are more prone to shear damage (*56,57*). In addition, cultures with a high portion of large cells generally are more viscous and may show higher yield stress owing to the higher culture solid content. Ballica and Ryu (*55*) observed the yield stress for *Datura stramonium, Pyrus communis,* and *L. erythrorhizon* to be dependent on PCV with an exponent of 4. Zhong et al. (*53*) observed expansion in individual cell volume during the batch cultivatioan of *Perilla frutescens.* Such cell enlargement resulted in increased PCV and led to a significant increase in apparent culture viscosity in the later stages of cultivation, despite the decrease in biomass concentration. A similar phenomenon was observed in batch cultures of *A. officinalis* supplemented with 5% sucrose (*13*). Supplying the medium with both 5% mannitol and 5% sucrose gave rise to an initial medium osmolarity of ca. 500 mOSM/L (cf., 250 mOSM/L with 5% sucrose). There was essentially no difference in dry weight and carbon utilization, but much lower values of PCV and apparent viscosity were observed compared to the batch culture with only 5% sucrose. The addition of mannitol caused apparent viscosity to be reduced by almost 84% (*13*).

Cell Aggregation, Foaming, and Wall Growth

Most plant cell suspension cultures have a mixture of aggregates over a wide size range. These aggregates are formed because of the failure of dividing cells to separate rather than clumping of previously formed single cells (*48*). Several concerns are associated with cell aggregation. The most obvious one is diffusion limitation and resulting nutrient (especially oxygen) concentration gradient in the aggregates. Diffusional limitation may create a stress for cells in the center of the aggregate to release metabolic byproducts. Locally high levels of such byproducts may alter the cell physiological state and may lead to organogenesis, or even become toxic to cells in the outer layers. Hulst et al. (*58*) estimated the maximum aggregate diameter without oxygen limitation in the *Tagetes patula* culture was about 3 mm. Aggregates of *T. patula* with a diameter larger than 5.4 mm showed cavitation in the center as a result of cell lysis. Another important aspect of cell aggregation is how cells associate with each other. Such cell–cell interactions may affect cellular differentiation and secondary metabolite production (*48*). Control of cell aggregation is difficult. The mild mixing intensity generally used in plant cell bioreactors is not intense enough to separate the cells in aggregates. The addition of sorbitol or cell-wall degrading enzymes such as pectinase and cellulase (*19*) or the reduction of calcium concentration in the medium has had limited success in the reduction of aggregate sizes in some culture systems (*59*). Higher cytokinin concentration has also been suggested to produce fine cell suspensions (*60*).

Foaming is frequently encountered in plant cell bioreactors with bubble aeration. For *A. officinalis* cultured in a bubble-aerated reactor, foaming was found most serious during early and later stages of cultivation. This is primarily owing to the initially high sugar concentration, and the presence of proteins released by lysed cells toward the end of cultivation. Silicone-based antifoam effectively controlled foaming in *A. officinalis* cell cultures without adversely affecting cell growth or rosmarinic acid production (*13,61*). Both silicone-based and polypropylene glycol antifoaming agents were successfully used in *C. roseus* cultures (*62*). However, reduced cell growth and secondary metabolite production by the addition of antifoams have also been reported (*63*). In addition, a decline in mass transfer may result from antifoam addition.

Wall growth resulting from cell floatation creates thick layers of necrotic cells built up on the reactor wall above the liquid surface, which may secrete byproducts such as proteases or superannuated cell organelles that can inhibit the cell growth (*64*). Wall growth can also affect scaleup and dynamic operating characteristics of chemostats (*54,59*) and bioreactor cultures with substrate inhibition (*65*). Reduction of wall growth in bench-scale glass reactors by coating the vessels with silicone-based water-repellent reagents (e.g., Sigmacote®) was generally unsuccessful (*13*). Control of culture foaming only reduces initial wall growth.

Once cells attached to the vessel wall, the meringue-like cell layer started to build up regardless the addition of antifoam. In fact, increased wall growth upon the addition of antifoam has been reported (*1*). In bench scale reactors, wall growth cells can be mechanically pushed back to the culture by means of a scraper unit to reach the entire periphery of the vessel (*13,66,67*). Wall growth can also be eliminated by using bubble-free aeration (*13,64,95,96*). Reduction of wall growth by lowering Ca^{2+} concentration in the medium has been reported by Sahai and Shuler (*67*).

Mixing

Mixing is important to keep the cells in suspension and provide a homogeneous environment to the cultured cells. The specific gravity of cultured plant cells is only slightly higher than the aqueous medium. For instance, the specific gravity of *C. roseus* and *Oryza sativa* are about 1.015 ~ 1.019 and 1.040 ~ 1.055 g/cm^3, respectively (*49*). However, because of their large size and formation of cell aggregates, cell sedimentation may occur as a result of insufficient mixing. For dense cell cultures, which have a high solid fraction (PCV > 50%), cell sedimentation is hindered (*68*). Yet, since the culture viscosity is high, suspension homogeneity hence becomes a primary concern in mixing (*69*).

For stirred tank reactors, an effective impeller design is of particular importance. The standard Rushton turbine has a number of disadvantages, including high power requirement, highly localized shear, and a drastic drop in power on aeration (*70,71*). This latter phenomenon is especially profound in viscous culture fluids, leading to poor pumping capacity (ability to circulate fluid elements). These limitations have motivated the use of other impeller types. Large-diameter impellers including helical ribbon impellers and helical screw (spiral) stirrers generally can provide high homogenization efficiency at low agitation speeds (*72,73*). By adding three vertical surface baffles, Jolicoeur et al. reported improved mixing in a plant cell bioreactor agitated by a helical ribbon impeller (*74*). A helical screw (spiral) stirrer was used in the cultivation of *Coleus blumei* (*75,76*). These impellers, however, suffer from their inefficiency in inducing bubble dispersion (*73*). A number of radial-flow impellers with a medium diameter including gate paddle impellers (*77*), large paddles (*78*), and hollow paddle impellers (*79,80*) have also been reported to give superior performance over the standard Rushton turbine in plant cell suspension cultures. Major limitations of these agitators are their poor efficacy in providing top-to-bottom blending and in inducing bubble dispersion (*71,72*). Recently introduced axial-flow hydrofoil impellers with large solidity ratios (the horizontally projected area of the blades/horizontal area swept out by the blades) have been reported to give efficient gas dispersion and bulk blending in highly viscous mycelial and gum fermentations (*70,81*). Flow and torque insta-

bility associated with this type of impeller can be reduced by operating at a upward rather than a downward pumping mode (*70,71*). The use of large solidity ratio hydrofoil impellers in high density plant cell cultures has yet to be tested.

For pneumatically agitated vessels, superficial gas velocity (and hence liquid circulation velocity) as well as reactor geometry can alter the suspension efficiency. Particle suspension generally is improved with a high reactor aspect (height-to-diameter) ratio, inclusion of a draft tube or a contoured bottom (*73*).

Global mixing quality in a bioreactor is usually characterized by measuring mixing time, which is defined as the time required to reach some arbitrary level of uniformity in the mixed fluid (*54*). For the same power input per unit volume, mixing time in airlift reactors is longer than in stirred tanks (*4,82*). Doran compared the performance of airlift and stirred tank reactors based on the time constants for oxygen conversion, oxygen transfer, and mixing, and arrived at a conclusion that mixing became the limiting factor for pneumatically agitated reactors at high plant cell concentrations (*4*). A number of studies have demonstrated that mixing problems developed in internal-loop airlift reactors at biomass densities above about 25 g dry wt/L (*57,80,83*). Mixing can be improved by replacing internal-loop air-lift reactors with external-loop reactors because higher circulation rates can be achieved in the latter (*82*).

Rotary drum reactors (RDR) have been used in several plant cell suspension culture systems with mostly satisfactory results (*84–86*). A 1000-liter RDR was successfully used by Takahashi and Fujita to culture *L. erythrorhizon* for the production of shikonin (*85*). No detailed mixing study, however, was conducted for this type of reactor.

Oxygen Demand and Supply

Cell respiration depends strongly on the culture conditions, data on the specific oxygen uptake rates for cultured plant cells have not been very consistent. According to Bond et al. (*62*), the maximum specific oxygen uptake rate for *C. roseus* cells was about 7.5 μmol/g dry wt/min, whereas only 0.4 μmol/g dry wt/min was reported in the study by Drapeau et al. (*87*). In general, oxygen demand for plant cells is considerably lower than for microbes (e.g., the maximum specific oxygen uptake rate for *E. coli* is about 260 μmol/g dry wt·min (*54*)). The pattern of oxygen uptake during the course of cultivation varies from species to species. For instance, the maximum oxygen uptake was observed near the onset of stationary phase in strawberry cell cultures (*88*), whereas for the *A. officinalis* culture, the peaks of oxygen uptake and mitotic index coincided with each other in the middle of the exponential growth phase (*89*). Oxygen demand has to be met so the dissolved oxygen level can be maintained above a certain critical concentration. The critical oxygen concentration for *C. roseus* and

D. carrata is 5×10^{-2} and 4.1×10^{-2} mmol/L, respectively (*5,90*). This is about 20% air saturation. Below the critical concentration, linear growth is usually observed due to oxygen limitation (*4*).

For a cell culture with a typical oxygen demand of 1 μmol/g dry wt·min, and a cell density of 10 g dry wt/L, a volumetric oxygen transfer coefficient (k_la) of 3/h is sufficient to maintain the dissolved oxygen concentration at 20% air saturation. Even for cultures with 10 times higher oxygen demand, the oxygen transfer requirement is still moderate. However, because of the high culture viscosity, cell shear sensitivity, and cell aggregation as discussed in the preceding sections, oxygen transfer in plant cell cultures becomes a challenging problem.

Stirred tank bioreactors with sparged aeration have been used extensively in plant cell cultures because of their superior mass transfer performance. A number of potential drawbacks, however, are associated with sparged stirred tank bioreactors. Bubble aeration induces foaming and wall growth. Over sparging may lead to stripping of volatile compounds such as carbon dioxide and ethylene (*66,91,92*). Moreover, bubble breakup in combination with mechanical agitation may contribute to cell damage, especially near the impeller region (*93*). In viscous culture suspensions, impeller configuration has a strong impact on k_la (*71*). A key performance indicator in choosing an appropriate impeller as a mass transfer device is the degree of power drop on aeration (*71*). Comparing with the Rushton turbines, the high solidity-ratio axial flow impeller described in the preceding section is able to maintain a higher power draw under high aeration, and hence gives a better oxygen transfer performance (*71,81*). As for the design of spargers, those control the bubble size at birth are by far preferable to spargers, relying on agitation dispersion, because less shear is generated (*94*). Therefore, a porpous sparger with an adequate pore size is preferred over a single point nuzzle sparger.

Stirred tank bioreactors with bubble-free aeration have also been investigated in plant cell cultures (*64,74,95*). The most apparent advantage of using bubble-free aeration in plant cell cultures is to eliminate culture foaming and wall growth (*13,64,95,96*). Bench scale plant cell chemostats may particularly benefit from this unique advantage. Large scale operation of bubble-free aeration, however, is limited primarily by the reduced specific aeration surface area (aeration surface per culture volume) during scale-up. The simplest bubble-free aeration method is surface aeration, which has been used in a helical-ribbon impeller bioreactor with surface baffles to culture high density *C. roseus* cell cultures (*74*). At 150 rpm (tip speed = 165 cm/s), k_la if ca, 40/h was achieved in water, but it decreased to < 10/h as the biomass concentration exceeded 25 g dry wt/L (*74*). Bubble-free aeration can also be achieved via diffusion of oxygen through silicone or microporous polymeric membranes immersed in cell cultures (*97*). *T. rugosum* was cultured in a 21-L membrane-stirrer reactor with bubble-free aeration (*64*). *In situ* stationary membrane tubing was used by Su and

Humphrey for the cultivation of *A. officinalis* in a bench-scale perfusion bioreactor (*13,95,96*).

For pneumatically agitated reactors, the main factors that effect $k_l a$ are superificial gas velocity, culture viscosity, and geometric parameters, such as the ratio of downcomer and riser cross-section area in air-lift reactors (*82*). In a recent study, $k_l a$ in an internal-loop air-lift bioreactor with plant cell cultures exhibiting pseudoplastic behavior and yield stress was found to be dependent on superficial gas velocity and apparent viscosity with an exponent of 0.3 and -0.4, respectively (*55*). Although higher oxygen transfer rates can be achieved by increasing the superficial gas velocity, this may lead to desorption of volatile compounds, foaming, and excess shear (*4,66,98,99*).

In order to study oxygen transfer without the complication of cellular oxygen consumption, a number of model culture fluids have been tested to simulate the rheological property of plant cell cultures. These include heat or chemical treated cell suspensions (*79*), and granulated agar suspensions (*52,57*). Heat or chemical treatments alter the cell morphological characteristics, and hence the rheological properties are different from the untreated cell cultures (*57*). Granulated agar suspension was first demonstrated by Tanaka to exhibit a similar rheological property as plant cell suspensions (*52*). This was recently verified by Jolicoeur et al. (*74*). The major drawback of this system is that it is not suitable for a large scale reactor, primarily because of the cost.

Shear

Cultured plant cells range from 20–40 μm in diameter and from 100–200 μm in length and embrace vacuoles up to 95% or more of cell volume (*100*). The primary cell wall contains parallel cellulose microfibrils embedded in a polysaccharide matrix. These features have led to the general belief that plant cells are shear sensitive (*49,56*). More evidence has shown, however, that the shear sensitivity of cultured plant cells strongly depends on the species (*56*). *N. tabacum* and *Cudrania tricuspidata*, for example, were found to be extremely shear sensitive (*52,101*). *Helianthus annuus*, on the other hand, can tolerate high shear (*51*). Even within one species, shear sensitivity varies. *C. roseus*, for instance, was found very shear-sensitive by Wagner and Vogelmann (*83*). Whereas in the studies of Scragg et al. (*51*), this species was found to be very robust. It is generally believed that older cells are more susceptible to shear damage than younger cells, such as those in the exponential phase. Markx et al. (*102*), however, found that shear sensitivity was only slightly affected by cell ages in cell suspensions of *C. roseus*, *N. tabacum*, *Cinchona robusta*, and *Tabernaemontana divaricata*.

In assessing the shear sensitivity of plant cells, it it essential to provide a suitable shear environment. Although cellular responses are easier

to interpret when a well-defined shear field is used, it is difficult to translate this information to the practical situation in a production fermenter. Meijer et al. (*56*) suggested that shear experiments should be conducted in a down-scaled version of the production vessel because of the resemblance of their shear environment, though the hydrodynamic shear fields in both vessels are not well defined. Meijer et al (*56*) also surveyed current techniques for characterizing culture responses to hydrodynamic shear. In technique proposed by Markx et al. (*102*), cytoplasmic membrane integrity was tested via measurement of the dielectric permittivity of cells at radio frequencies. When the cell membrane is damaged by shear, the ions cross the membrane freely, therefore reducing the capacitance of the cell to almost zero. According to Markx et al. (*102*), this technique can be used to assess shear damage at real time by measuring the capacitance of the cell culture.

Characterization of shear field in bioreactors is important not only from the perspective of ensuring a low shear environment for shear-sensitive cells, but also for estimating the apparent viscosity of non-Newtonian culture fluids in the reactor. In characterizing the shear intensity in cell culture bioreactors, both average and local shear should be considered. In estimating the average shear in stirred tank reactors, the classical approach by Metzner and Otto (*103*) has been used most frequently. In this approach, the average shear rate (γ_{ave}) is linearly correlated with the impeller speed (N), i.e.,

$$\gamma_{ave} = k_s \cdot N,$$

where k_s is a constant depending mainly on the impeller type. A similar linear correlation has been derived by Nishikawa et al. for pneumatically agitated reactors (*104*). In this correlation, the average shear rate is linearly related to the superficial gas velocity. The values of the proportionality constant, however, vary considerably in the literature (*4*). Tanaka used the solid–liquid mass transfer coefficient, obtained from measurement of the solid dissolution rate, as an index for average shear (*52,57*).

For the measurement of local shear rates at the impeller blades and at the vessel wall, the electrochemical technique has been used by a number of investigators (*105,106*). In this method, the limiting current, controlled by the diffusion of ions to small electrodes, was measured and correlated with hydrodynamic shear. Kim et al. (*107*) recently used a dissolved oxygen (DO) probe to measure local shear rates in a cell culture reactor by correlating the shear rate with the thickness of the liquid film surrounding the DO probe membrane. Although very easy to use, the sensitivity of this technique is doubtful. Moreover, both liquid velocity and hydrodynamic shear (caused by the velocity gradient) affect the liquid film thickness and these effects usually can not be distinguished.

Shear-induced cell death has been correlated with bioreactor operating parameters for animal and insect cell cultures. For a nonsparged

stirred tank reactor, according to a turbulent-eddy model, the shear-induced specific cell death rate (q_d) is expressed by the following equation (*108*):

$$q_d = k_e [P/(\rho V \nu^3)]^{0.75} = k_e[(N_p N^3 D_i^5)/(g_c V \nu^3)]^{0.75} \quad (2)$$

where k_e is a proportionality constant, P is ungassed power dissipation, ρ is the density of culture fluid, V is culture volume, ν is kinematic viscosity, N_p is power number ($= P \cdot g_c/\rho N^3 D_i^5$), D_i is the impeller diameter, and g_c is the gravitational conversion constant. According to this equation, a low power number impeller with a smaller diameter should be used to reduce the hydrodynamic shear. Moreover, shear level should remain constant if scale-up is based on a constant power per unit volume. For a sparged stirred tank reactor, shear-induced cell death is more complex. A cell–bubble interaction model was proposed by Yang and Wang (*93*) to account for cell inactivation in the presence of sparging and mecahical agitation. According to this model, the specific death rate is linearly related to the specific bubble interfacial surface area.

For pneumatically agitated vessels, bubble breakup is believed to be the culprit for shear-induced cell death. The specific death rate of animal or insect cells caused by bubble bursting can be estimated using the following equation (*109*):

$$q_d = \{3\Psi \cdot \delta[P_g/(\rho g V)]/[d_b \cdot h_l]\} \quad (3)$$

where Ψ is equal to $\phi \cdot X_f/X_b$ (ϕ is the fraction of cells killed in each thin film surrounding a bursting bubble, X_f and X_b are cell concentration in the film and in the bulk liquid, respectively), δ is the film thickness, P_g is the gassed power input, g is the gravitational acceleration, d_b is the average bubble diameter, and h_l is the height of the culture fluid. Based on this equation, shear problem should be reduced in large tall reactors when the power input per unit volume is kept constant during scale-up. Because of the differences in cell sizes and structures (e.g., cell wall) between plant and animal cells, the applicability of Eq. (3) in plant cell cultures has yet to be verified.

Bioreactor Operation Strategy

A number of operating strategies can be applied in plant cell bioreactors. In addition to the standard batch cultivation, there are multistage batch, fed-batch, single-, or multistage continuous (chemostat), semicontinuous (draw-and-fill or repeated batch), and perfusion (chemostat with cell retention) cultivation. An effective bioreactor operating strategy should provide a high productivity, a high product yield (product formed per substrate consumed), and a high product content (product/cell wt). Higher productivity means more product can be formed per unit time per

unit reactor volume. High product content makes downstream separation and purification easier. High product yield reduces the cost for substrates. Generally, the operating strategy is determined based on the pattern of product formation and the way the product is translocated following its synthesis (i.e., whether the product is excreted into the medium or retained in the cell). It is very common to relate the pattern of product synthesis to cell growth. That is, product synthesis is frequently characterized as growth associated or nongrowth associated (*5*). One should be cautious, however, to select an appropriate index for cell growth. For example, rosmarinic acid production in a perfused *A. officinalis* culture followed a growth-associated pattern with respect to cell dry weight, but this pattern was not observed if the cell growth was expressed as cellular protein increases (*61*). This is perhaps because of the increased sugar storage during culture perfusion, leading to a dry weight increase that is not parallel to cell division.

Growth-Associated, Intracellular Products

High productivity of secondary metabolites can be achieved by increasing the cell growth rate in a single-stage bioreactor. Since the product is stored within the cell, a culture strategy that gives a high biomass output rate is most desirable. In this respect, a continuous cultivation is more attractive than a batch one (*54*). However, it is difficult to run a chemostat with plant cell suspensions because of cell aggregation, slow growth, surface adhesion, and high viscosity at high cell densities. Semicontinuous cultivation can be applied as an alternative to chemostat cultures. In semicontinuous cultures, a portion of the cell suspension is periodically removed and then replenished with fresh medium. The amount of culture removed each time needs to be determined carefully. This is because the cells remained in the reactor after each culture removal act as an inoculum for the subsequent production period, and inoculum size is known to have a strong impact on cell growth and metabolite production in plant cell cultures (*19,61,110,111*). Semicontinuous cultivation has been used in several plant cell systems, including the biotransformation of β-methyldigitoxin to β-methyldigoxin (*112*), ginseng biomass production (*113*), and recently in the anthocyanin production by *Aralia cordata* (*114*).

Besides semicontinuous culture, perfusion culture with a bleed stream deserves special attention. In this case, while most of the cells are retained in the bioreactor, a portion of culture suspension is constantly or periodically removed from the reactor. A much higher cell density can be obtained in perfusion cultures compared to continuous or semicontinuous cultures, because cells are retained within the reactor via a cell retention device. With a bleed stream, the perfusion reactor can be operated under a steady state at a very high cell concentration. For a culture system that follows simple Monod kinetics, the maximum biomass output rate in a perfusion reactor with a bleed stream is higher than that in a chemostat

by a factor of $1/\beta$, where β is the bleed ratio (the ratio between flow rates of the bleed stream and the feed stream). The specific growth rate in the perfusion reactor can be manipulated by adjusting the flow rate of the bleed stream. Furthermore, since very high perfusion rates can be used without cell washout, inhibitory byproducts in the spent medium can be removed efficiently. Perfusion culture has been used in the commercial production of berberine, an intracellular, growth-associated metabolite, by Mitsui Petrochemical Industries, Ltd. (*2,5*). Kim and coworkers also used perfusion cultivation in the production of berberine (*115*). Recently, a high cell density of 35 g dry wt/L and a rosmarinic acid concentration of 4 g/L were achieved in the perfusion culture of *A. officinalis* (*61*).

Growth-Associated, Extracellular Products

Perfusion culture with a bleed stream is also very useful. To increase the product output, perfusion culture should be operated at a high perfusion rate with the bleed stream adjusted to give a high specific growth rate.

Nongrowth-Associated, Intracellular Products

Two stage batch culture method has been advocated in many plant cell systems primarily because of the success of the shikonin process (*2,21,85*). In this method, two media are used. In the first stage reactor, a growth medium is utilized to increase cell concentration. Cells from the first stage are then concentrated and inoculated into the second stage reactor containing a medium that stimulates product formation. Since cell growth is generally repressed in the second stage reactor, a high inoculum size is needed to improve the productivity. However, high inoculum size may reduce the inducing or synergistic effect of the production medium, as shown in the rosmarinic acid production by *A. officinalis* (*61,111*) and ajmalicine production by *C. roseus* (*110*). In this latter case, lower activities of major enzymes in ajmalicine synthesis were observed when a high inoculum size was used with the production medium.

Two-stage chemostat may also be considered. It has been used by Sahai and Shuler in the study of phenolic production by *N. tabacum* (*67*). The idea was to use the first stage chemostat to provide the cells for the second stage chemostat, which is manipulated to enhance product synthesis. A low dilution rate should be used in the second-stage chemostat to reduce the cell growth rate. This is usually done by increasing the reactor volume of the second stage chemostat. One major drawback of this operation is that the low dilution rate also reduces the biomass output rate and hence decreases the metabolite productivity.

Nongrowth-Associated, Extracellular Products

Perfusion culture is an obvious choice (*5,17,61*). The reactor can be operated at a very high cell density without rapid cell division for a long period. The product can be continuously harvested from the spent medium. For cultures limited by nutrient depletion and/or accumulation of growth inhibitors, perfusion culture is especially useful. It can be

operated for a prolonged period with a constant supply of fresh nutrient and removal of spent medium that might contain inhibitory materials.

Cell Retention in Perfusion Bioreactors

In designing a cell retention device for plant cell perfusion cultures, factors to be considered include solid content and viscosity, cell shear sensitivity, aggregate size distribution, as well as scaleability. Currently, cell–medium separation in perfusion reactors is based on either sedimentation or filtration.

Cell retention by sedimentation is done by incorporating an *in situ* or *ex situ* settler (or a continuous centrifuge) with a bioreactor (*115–125*). *Ex situ* settlers are not suitable for high density plant cell suspensions since continuous circulation of viscous, shear sensitive culture suspension through an external loop can be problematic. On the other hand, *in situ* settler, in the form of a settling column, has been used in perfusion cultures of *C. roseus* and *T. rugosum* (*115,119*). In these studies, the settler was in immediate contact with the turbulent mixing zone in the reactor. There was no means to direct the cell particles away from the settler. Therefore, there were always a large number of cells trapped in the settler especially under high perfusion rates. Also, bubbles might get into the settling zone to disturb cell sedimentation. Recently, a perfusion bioreactor was developed to overcome these problems (*126*). In this reactor, a settling zone was created within the downcomer section of an external-loop air-lift reactor. By taking advantage of the well-defined flow pattern in the air-lift reactor, the downward bulk liquid flow in the downcomer can guide the cell particles away from the settling region so that only few cell particles are present in the settler. In addition, since gas hold-up in the downcomer is less than in the riser, there is less chance of bubbles getting into the settling zone. As a result, complete cell retention can be achieved at very high perfusion rates.

Cell–medium separation by filtration can be achieved by using stationary or moving filters. A stirred tank reactor with *in situ* filtration using a stationary stainless steel filter ([5.1] in Fig. 1) was used to culture *A. officinalis* to high cell density (*13,95,96*). Payne et al. (*127*) conducted a two-stage batch cultivation in a bubble column that coupled aeration to filtration. Medium exchange was done via filtration using a cheesecloth filter that also served as a gas distribution in the bubble column. The most common moving filter is the spin filter, i.e., a cylindrical filter attached to the agitator shaft (*128*). Spin filter technology has been employed in somatic plant embryo cultures (*129*). The centrifugal force generated via the rotation of the cylindrical filter is expected to hinder filter clogging. However, it has been shown in a recent study (*130*), that high spinning rates also promote fluid exchange across the filter, and therefore reduces particle retention efficiency. Obviously, there is a trade-off by using high rotation speed to prevent filter fouling. The spin filter method is not par-

ticularly suitable for viscous plant cell suspensions owing to the difficulty of preventing filter fouling. In addition to spin filters, Wagner and Lehmann (*131*) have installed hydrophilic microporous polypropylene membrane fibers inside the reactor as a microfilter-membrane stirrer to retain and mix animal cells in a bioreactor.

In Situ Extraction

Creating an artificial storage compartment outside the cells may improve the production of secondary metabolites that already show some degree of secretion into the medium. This has been done by the addition of a solid or water-immiscible liquid phase to trap excreted products in two-phase cultures (*132*). Various ion exchange resins have been employed as the extractive phase. Amberlite XAD-7 was used to adsorb indole alkaloids, including ajmalicine and serpentine, from *Catharanthus roseus* culture (*133*) and sanguinarine from *Papaver somniferum* culture (*134*). A number of water-immiscible liquid phases have also been used. Miglyol, a liquid triglyceride of low viscosity composed of fatty acids with 8–10 carbons, has been added at 10–12% (v/v) in cell suspensions of *N. tabacum* (*135*) and *Matricaria chamomilla* (*132*) as the extractive phase to improve accumulation of secondary substances without a reduction in cell growth. Liquid paraffin (10% v/v) was also used in *M. chamomilla* cell culture for adsorption of lipophilic secondary products (*132*). Byun et al. (*136*) used a dimethyl siloxane polymer (a silicone-based antifoam solution) as the second phase. They found selective accumulation of high levels of benzophenanthridine alkaloids in the silicone phase. The best result was obtained at ca. 23% (v/v). Decreases in cell growth were observed at silicone fluid concentrations higher than 23% (*136,137*).

In using the two-phase cultures, several limitations should be realized. First, the introduction of a second liquid phase made of lipophilic organics or dimethyl siloxane polymers, generally at high volume percentages (10–20% v/v), may cause a dramatic reduction in oxygen transfer. In addition, for those secondary products that prefer vacuoles or cellular organelles as their storage compartments, introducing an extracellular artificial storage site would not be very useful since there is no suitable transport mechanism to direct the efflux of secondary metabolites. To overcome the first limitation, a cell-recycle or perfusion reactor can be coupled with an external column extractor. In this case, secondary metabolites in the cell-free aqueous spent medium can be continuously extracted into an accumulating phase outside the reactor. Higher extraction efficiency can also be expected, since better mass transfer can be achieved with the external extractor and there is no direct contact of the extractive phase with the cells in the reactor. As for the second limitation, inducing product secretion by chemical means, such as membrane permeabilization using dimethylsulfoxide (DMSO), has been tested in a number of culture systems (*138*). The premise for successful chemical

permeabilization is that cell viability should be preserved and membrane translocation should be the bottleneck for metabolite efflux. Brodelius suggested that loss of cell viability typically associated with chemical permeabilization was most likely not caused by the toxicity of the permeabilization reagent, but rather to the destruction of cell compartmentation and the subsequent release of intracellular toxic substances, such as proteases and phenolics (*30*). In a recent study, Park and Martinez (*139*) reported improved rosmarinic acid secretion with preserved cell viability by preadapting *C. blumei* in media containing gradually increased DMSO concentrations. It is not clear, however, whether this technique is applicable to other plant cell cultures. In *Cinchona ledgeriana* cultures, although viability was largely preserved after DMSO treatment, only a small amount of quinoline alkaloids were released (*140*), suggesting that the bottleneck for efflux was not membrane translocation. Compared to chemical permeabilization, elicitation appears to be a more promising technique for stimulating the secretion of secondary metabolites (*30,38,43*). Brodelius suggested that the improved secretion of secondary products in elicited cultures might be a result of the enhanced expression of carrier proteins (*30*).

PROGRESS IN COMMERCIAL DEVELOPMENT

Since the introduction of the shikonin process by Mitsui Petrochemical Industries, Ltd. of Japan in 1983, progress on plant tissue cultures that shows commercial potential has been made in both academia and industry. Japan is still the leading country in the commercial development of plant cell cultures. In a recent publication "Plant Cell Culture in Japan" (Komamine, A., Misawa, M., and DiCosmo, F., eds., CMC, Tokyo, 1991), some of the research activities in the commercial production of phytochemicals using plant cell culture technology by Japanese exterprises were described. These and other major activities are summarized as follows.

In order to stimulate the commercial development of plant cell cultures in Japan, Plant Cell Culture Technology Inc. was founded in 1987 with a capital of ca. $10 million (*141*). The total research and development cost for 6 yr was about $20 million, whereas funding was provided by the Japanese government (70%) and seven companies (30%). The companies are Kirin Brewery, Kodama Hakko, Mitsui Petrochemical, Mitsui-Toatsu Chemical, Hitachi, Suntory, and Tonen; active research is being carried out in the research laboratories of these companies. One recent effort by the P. C. C. was the large-scale (500 L) production of anthocyanins using a dark-grown *Aralia cordata* cell suspension culture (*114*). During the scale-up of this process, desorption of volatile components from the liquid medium owing to air sparging was found to be detrimental to cell growth and

anthocyanin production. By supplementing carbon dioxide in the inlet gas stream, and using a semicontinuous culture technique, a successful large-scale cell culture process was developed.

Being the first commercial plant cell culture process, shikonin production by *L. erythrorhizon* has received wide publicity since its debut in 1983. In this process, a two-stage culture method was used, with the second stage (the production stage) operated at high cell density using a fed-batch technique. A rotating drum fermenter was found to be superior to the conventional stirred tank or air-lift type fermenters. High shikonin producing cell lines were established based on protoplast selection (*85*). A similar cell line selection method was used for selecting the high berberine producing *Coptis japonica* cell lines (*142*). In the berberine process developed by Mitsui Petrochemical Industries Ltd., *C. japonica* was cultivated in a high density perfusion culture with constant cell harvest (*142*). In 1988, the commercial production of ginseng and saponin from *Panax ginseng* cell cultures was initiated by the Nitto Denko Co. (*3*). The product is added to wines, tonic drinks, soups, herbal liquors, and other preparations. Saponin can also be used as an antiulcer agent. In the commercial ginseng process, an embryo-like cell line with high proliferation rates is cultured in a 2000-L fermenter. The maximum biomass productivity is approx 700 mg/L/d.

Ikeda and coworkers at the Japan Tobacco Inc. have investigated the production of *Mirabilis* anti-plant-viral proteins (MAP) by *Mirabilis jalapa* cells in suspension culture (*143*). MAP is very effective in preventing viral infection, especially by contact-transmitted viruses such as the tobacco mosaic virus. High MAP producing cell lines have been selected. However, MAP content in the cultured cells decreased as the culture was scaled up (*143*). Nippon Paint Co. has engaged in a variety of research activities involving plant cell cultures. Their research efforts include production of lichen substances as antibiotics, antioxidants and dyes by in vitro lichen cultures (*144*), pigment and quercetin glucuronide production by *Euphobia millii* cell cultures, and production of a blood coagulation component by cultured plant cells of the *Hydrocotyle* genus (*see* the patent section). Shiseido Co. has developed a biotransformation process for the production of arbutin, a skin depigmenting agent, by *Catharanthus roseus* cell cultures (*145*). In this process, glucosylation of hydroquinone to form arbutin was catalyzed by uridine diphosphate (UDPG)-glucose: hydroquinone glucosyltransferase of the *C. roseus* cells. Nippon Oil has established a process for the production of podophyllotoxin, an intermediate of the antitumor drugs, etoposide and teniposide (*141*).

Compared with Japan, commercial development of plant cell cultures in the United States and Europe has been less active. There are few notable commercial exploitation. Large-scale production of phosphodiesterase by Bethesda Research Laboratories in the United States was cited in an article by Buitelar and Tramper (*146*). Recently, a consortium was

formed (members including Cornell University, USDA-ARS, Phyton Catalytic, Hauser Chemical Research, and Colorado State University) with the goal of gaining a broad-based understanding of manipulation of tissue cultures of *Taxus brevifolia* and other related species to produce taxol or taxol-like compounds (*147*). In 1991, a US patent was awarded to Escagenetics Inc., a California-based agrobiotechnology company, for the production of vanillin by vanilla callus suspension culture. The same company is also pursuing the production of taxol by callus cultures (*148*). Use of differentiated nodule tissues in suspension cultures for the production of taxol and taxanes (taxol precursors) was recently proposed by researchers at the University of Wisconsin (*149*). Nodules are independent, spherical, dense cell clusters that form a cohesive unit and display a high level of cellular organization. Production of ribosome inactivating proteins that exhibit antiviral (including HIV-1) and anticancer activities from cell suspension or hairy root cultures of *Trichosanthes kirilowii* was explored by researchers at the University of California at Davis (*150*) and Pennsylvania State University (*151*). In Germany, a five-stage fermentation plant for plant cell cultivation, with the largest reactor at a scale of 75,000 L, has been reported (*152*). According to Westphal (*152*), large amounts of immunologically active polysaccharides were produced by cell cultures of *Echinacea purpurea* using this facility. Other activities in the commercial exploitation of secondary metabolite production in plant cell cultures can be seen in the patent section.

CONCLUSION AND OUTLOOK

This work has discussed physiological as well as bioreactor operating characteristics of plant cell suspension cultures. It is difficult to generalize the complex physiological responses expressed in different culture systems upon a certain treatment. Some methodologies, such as elicitation and hormone treatment, however, have proven effective to enhance secondary metabolite production in many culture systems. Elicitation, in particular, is of considerable interest because it also promotes product excretion. Culture rheological properties, especially the yield stress behavior, should be carefully examined since these properties have a very profound impact on mixing and oxygen transfer. Mechanisms for shear-induced inactivation of primary and secondary metabolisms ought to be further researched. As for bioreactors, most studies have focused on oxygen transfer. Suspension homogeneity has received much less attention, although it strongly affects the culture microenvironment, especially in large-scale cultures at high cell densities. Perfusion cultivation has offered a number of unique advantages for plant cell cultures. Its application in secondary metabolite production merits further studies. In many ways, suspension culture is a very promising technique and certainly deserved more research to fully explore its potential for secondary metabolite production.

PATENTS AND LITERATURE

Patents

In this section, representative patents related to the use of plant cell suspension cultures for the synthesis of natural compounds are described. This list is not intended to cover all the patents pertinent to the searched subject. However, this list should reflect major recent developments in industrial and academic sectors.

Both US and Japanese patents were searched using the database available at the Hawaii State Library. The time period covered in the US patent search is from 1988 to 1993. The Japanese patent search only covered from 1988 to 1990, owing to the unavailability of the more recent patent database at the Hawaii State Library. For the US patents, fields of search include 435/240.46 and 435/240.48, which comprehend patents related to plant callus or cell suspension cultures. For Japanese patents, the major search heading was "callus." The major patents recovered under this search are described below. Abstracts are included for the US patents, whereas a large number of Japanese patents were found under this search and hence only brief remarks are included for clarity.

US Patents

Hara, Y. and Suga, C.
Method for producing secondary metabolites of plants
US Pat. 4,717,664, Jan. 5, 1988
Assignee: Mitsui Petrochemical Industries Ltd., Japan

This invention provides for a method of producing secondary metabolites of higher plants, such as *Lithospermum erythrorhizon, Coptis japonica,* and *Nicotiana tabacum,* by suspension cultures of a mass of undifferentiated cells (callus). The cultures were carried out in at least two stages in liquid media. The first-stage culturing is carried out in a liquid medium conventionally used for the tissue culture of plants, which contains indispensably inorganic substances and carbon sources and additionally, phytohormones, vitamins, and/or amino acids. Whereas the second-stage culturing is carried out in another liquid medium, of which the concentration of at least one of the constituents is substantially varied, namely decreased or increased, from the first-stage liquid medium. According to such two-stage liquid medium cultures, the productivity of the secondary metabolites, such as shikonin, berberine, and nicotine, are significantly increased, even though the rate of cell growth in the second stage is relatively low.

Weathers, P. and Giles, K.
Mist cultivation of cells
US Pat. 4,857,464, Aug. 15, 1989
Assignee: Bio-Rational Technologies Inc.

Cells are cultivated in a chamber having a high nutrient humidity. Gas drives liquid nutrient through devices that produce a fine mist within the chamber. Screens or convoluted mesh supports the cells, and provides for drainage of cell products and media away from the cells for processing. The chamber is sealable to provide for axenic conditions.

Yamamoto, Y., Mizuguchi, R., and Shibata, T.
Plant culture cell and use thereof
US Pat 4,970,151, Nov. 13, 1990
Assignee: Nippon Paint Co. Ltd., Japan

Disclosed is culture plant cell derived from tissues or cells of a plant belonging to *Hydrocotyle* genius (water pennyworts) and *Centella* genus, a culture method thereof, and a blood coagulation component and a therepeutic agent for mental disease obtained from the cultured plant cells.

Furuya, T.
Process for production of tocopherols
U.S Pat. 4,978,617, Dec. 18, 1990
Assignee: Showa Denko Kabushiki Kaisha, Japan

A new process for the production of tocopherols by tissue culture, which comprises

1. Preparing a callus of a plant *Carthamus tinctorius;*
2. Inoculating the callus to produce tocopherols; and
3. Recovering the tocopherols.

The tocopherols thus produced are mainly α-tocopherol, which has the strongest vitamin E activity among tocopherol analogs.

Christen, A. A., Gibson, D. M., and Bland, J.
Production of taxol or taxol-like compounds in cell culture
US Pat. 5,019,504, May 28, 1991
Assignee: The United States of America as represented by the Secretary of Agriculture

Tissue of *Taxus brevifolia* has been successfully cultured to produce taxol, related alkaloids, and alkaloid precursors. These procedures will provide a supply of chemotherapeutic agents.

Petiard, V. and Yvernel, D.
Process for cultivating plant cells in vitro
US Pat. 5,030,573, Jul. 9, 1991
Assignee: Nestec S. A., Switzerland

Plant cells are cultivated in vitro without agitation or fixation, e.g., for the production of a metabolite or a biotransformation. The liquid medium may be circulated for removal of the desired products.

Vunsh, R. and Matilsky, M. B.
Production and use of a high-intensity red natural colorant derived from carrot cell tissue cultures
US Pat. 5,039,536, Aug. 13, 1991
Assignee: International Genetic Sciences Partnership

The production and use of a high-intensity red natural colorant prepared from an anthocyanin derived from the cell line of *Daucus carota* (carrot) is described. The production of the high-intensity red natural colorant comprises the cultivation of *Daucus carota* in a cell tissue culture. Carrot cells are extracted after a suitable growth period. The extract is then purified in order to isolate the anthocyanin found in the carrot cells. The anthocyanin is then concentrated to produce the red natural colorant of the invention. The natural colorant produced is stable over a wide pH range under various conditions. The natural colorant is useful as a coloring agent for food products, cosmetics, and pharmaceuticals.

Knuth, M. E. and Sahai, O. P.
Flavor composition and method
US Pat. 5,057,424, Oct. 15, 1991
Assignee: Escagenetics Corporation

A vanilla composition is produced by vanilla plant callus cells suspended in tissue culture, under conditions that promote secretion of vanilla flavor components into the culture medium. The flavor components may be separated from the medium by adsorption resins. Also disclosed are methods for preparing callus cells capable of secreting flavor components in tissue culture, and callus cells produced thereby.

Reuther, G. R.
Process for the preparation of pilocarpine from in vitro cultures of pilocarpus
US Pat. 5,059,531, Oct. 22, 1991
Assignee: Merck Patent Gesellschift Mit Beschrankter Haftung, Germany

Pilocarpine is isolated from *Pilocarpus* from suspension cultures or in vitro cultures of differentiated plants, where, to establish the in vitro cultures, specific culture media are used that differ in their hormone composition and that are used in a particular sequence.

Murata, Y., Otsuka, M., Saimoto, H., and Kawashima, M.
Process for the production of betacyanin pigments
US Pat. 5,089,410, Feb. 18, 1992
Assignee: Somar Corporation, Japan

A biotechnological process for the production of betacyanin pigments is disclosed that comprises cultivating calli, induced from a plant that belongs to *Beta vulgaris* L. and that is capable of producing betacyanin pigments, in a liquid culture medium containing a reducing agent such as glutathione.

Clarke, A. E., Bacic, A., and Lane, A. G.
Plant gum material and use thereof in food products
US Pat. 5,133,979, Jul. 28, 1992
Assignee: Bio Polymers Pty. Ltd. and Commonwealth Scientific and Industrial Research Organization, Australia

A method for the production of plant gum product that comprises the steps of culturing gum-secreting plant cells in tissue culture in the presence of a culture medium; and recovering the gum product secreted by the cells from the culture medium. The gum product may be used in a food product, for example, as an emulsifying or stabilizing agent.

Kinnersley, A. M. and Henderson, W. E.
Method and composition for plant tissue and cell culture
US Pat. 5,153,130, Oct. 6, 1992
Assignee: CPC International Inc.

Improved secondary metabolite production is achieved by culturing plant tissue and plant cells in a culture medium wherein the carbohydrates comprise a mixture of maltose and glucose.

Yamamoto, Y. and Kinoshita, Y.
Production of quercetin glucuronide
US Pat. 5,212,076, May 18, 1993
Assignee: Nippon Paint Co. Ltd., Japan

The present invention provides a dye other than red and purple that is obtained from cultured cells of *Euphorbia millii*. The present invention also provides cultured cells containing quercetin glucuronide in a large amount, derived from tissues or cells of *Euphoribia millii*.

Japanese Patents, 1988

Pat. no.	Title of invention	Assignee	Remarks
63,123,386	Production of plant virus inhibitor	Japan Tobacco Inc.	*Mirabilis jalapa* cells
63,199,766	Method of increasing yield of red pigment of safflower	Kibun K.K.	Medium w/o calcium or magesium
63,207,379	Production of differentiation agent for plant callus cell	Nok Co.	From culture of *Enterobacter* sp.
63,207,380	Production of cultured plant cell containing antioxidative substance	Kobe Steel Ltd.	Sesame culture
63,207,389	Production of lignin compound by cultured cell	Kobe Steel Ltd.	Sesame culture
63,216,478	Preparation of tissue culture product of Brazilian carrot	KK Japan Bio Kenykusho	Products include saponin and β-ecdysone
63,233,782	Method for inducing plant or plant callus capable of high peroxidase production	Nissin Food Prod. Co. Ltd.	Medium contains methyl-viologen or its analog
63,233,993	Production of red pigment	Nitto Electric Ind. Co. Ltd.	Anthoclyanins from *Ipompea batata*
63,240,795	Purple pigment and production thereof	Nippon Paint Co. Ltd.	Anthocyanins from *Euphorbia millii*
63,251,092	Production of albumin	Shiseido Co. Ltd.	Biotransformation of hydro-quinone by *C. roseus*
63,258,589	Production of colchicine or demecolcine by tissue culture of *Colchicum*	Ajinomoto Co. Ltd.	Colchicine: for acute gout; demecolcine: for myeloid leukemia
63,317,090	Production of plaunotol	Kirin Brewery Co. Ltd.	Antiulcer agent from croton cell culture

Japanese Patents, 1989

Pat. no.	Title of invention	Assignee	Remarks
01,102,027	Production of antiallergic drug	Nitto Kenko Co.	Cell culture of the genus *Lauraceae* or *Labiatae*
01,113,320	Agent for suppressing blood pressure increase	Kao Co.	Callus culture of *Musci* or *Hepaticae*
01,151,504	Production of nematocide	Shikoku Chem. Co.	Marigold cell culture
01,218,582	Culture of herb plant tissue	Mitsubishi Heavy Ind. Ltd.	Production of essential oil
01,218,591	Production of antimicrobial substance	Noyaku Biotechnology	By inoculation of *Piricularia oryzae* spore suspension into rice cell culture
01,218,600	Production of 7-hydroxytesterone	JGC Co.	Hydroxylation of testosterone by *Marchantia* cell culture
01,230,525	Production of antiulcer agent	Nitto Denko Co.	Cell culture of a pea family plant
01,289,492	Production of allilin or methylcysteine sulfoxide	Ajinomoto Co. Inc.	Biotransformation of allyl-cysteine or allylmercaptane by garlic cell culture

Japanese Patents, 1990

Pat. no.	Title of invention	Assignee	Remarks
02,013,390	Production of tabersonine derivative	Tao Nenryo Kogyo KK	Useful as an intermediate for biosynthesis of vincristine
02,057,189	Production of berberine by vapor culture of *Thalictrum minus L. var hypoleucum Miq.*	Nitto Denko Co.	Medium is sprayed onto the callus
02,124,098	Production of indole alkaloid	Mitsui Petrochem. Ind. Ltd.	Vinblastine from *C. roseus* cell culture
02,171,191	Production of yellow substance and use thereof	Daiso Co. Ltd.	Cell culture of *Corydalis ambiqua*
02,172,921	Production of antitumor agent	Nitto Denko Co.	Inulin from cell culture of *Platycodon grandiflorum*
02,191,292	Production of acteoside	Tsumura and Co.	From cell culture of the genus *Leucosceptrum*
02,211,891	Production of phododendrol glycoside using tissue of *Acer nikoense maxim.*	Snow Brand Milk Prod. Co. Ltd.	For liver disorder
02,215,397	Production of panaxsaponin by tissue culture of *Panax japonicus*	Nitto Denko Co.	As an antiulcer agent
02,215,721	Anticancer agent	Kao Co.	Acidic heteropolysaccharides from cell culture of the genus *Polianthes L.*

(*continued*)

02,234,696	Production of antiulcer agent	Nitto Denko Co.	Saponin from *Panax japonicus* cell culture
02,242,691	Production of betacyanin-based dyestuff by cultured cell	Sumar Co.	*Beta vulgaris*
02,265,475	Red dye and production of callus containing same	Nippon Shokubai Kagaku Kogyo Co. Ltd.	*Raphanus sativus*
02,268,678	Production of callus containing cycosaponin	Mitsui Toatsu Chem. Inc.	*Bupleurum falcatum*
02,268,698	Judgment at initial stage of logarithmic propagation in plant tissue culture	Nitto Denko Co.	Based on peroxidase activity
02,276,580	Production of red coloring matter from cultured safflower cell	Mitsui Eng. and Shipbuild Co. Ltd.	Cytokinin conc. ≥ auxin conc.
02,276,581	Production of red coloring matter from cultured safflower cell	Mitsui Eng. and Shipbuild Co. Ltd.	Redifferentiation of calli into petals
02,276,582	Production of red coloring matter from cultured safflower cell	Mitsui Eng. and Shipbuild Co. Ltd.	Release of pigment into an alkaline medium

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