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Shear Sensitivity of Plant Cells in Suspensions

Present and Future

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ABSTRACT

Plant cells are a source of pharmaceuticals, fragrances, flavors, and dyes that are traditionally produced by extraction of tissues from whole plants. Recent trends in plant product research, transformed cell lines, and conservation policies place increased demand on plant cell culture technology. Unlike processing of microbial and animal cells in bioreactors, no economically viable process based on the suspension culture of plant cells in bioreactors has yet been possible in North America. It is proposed that the suspended-cell bioreactor is the method of choice and that plant cells respond to fluid forces (defined as laminar shear and turbulent eddies-based and bubble-based forces) differently from their animal cell counterparts in bioreactors. Although plant cells produce a tough cell wall, fluid forces, although not lethal within normal range, impact the membrane transport processes and metabolic function of plant cells; these effects are termed sublytic. Previous approaches to shear sensitivity of plant cells are reviewed in the context of these sublytic effects. A model for systematic evaluation of fluid-mechanical causes and physiological mechanisms behind sublytic effects is proposed. It is further proposed that, once understood, the plant cell's sublytic responses to fluid force can be used advantageously in stirred suspension cultures.

Index Entries: Plant cells; bioreactor; shear sensitivity; sublytic effects; taxus; carrot; turbulence.

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INTRODUCTION

Plant cells are a source of more than 30,000 different chemicals that are traditionally produced by extraction of tissues from whole plants to manufacture pharmaceuticals, dyes, flavors, and fragrances. For example, more than 25% of all prescription drugs in the USA are derived from plants. With the success of Taxol in clinical trials for cancer treatment and an inadequate supply of Yew trees (*Taxus*), there is a renewed interest in large-scale plant cell culture technology (*1*). Suspension culture of plant cells, analogous to microbial and animal cell cultures, offers an alternative route to production of some of these chemicals produced from plants or even new products that are not found in whole plants. Plant cell culture methods are also being proposed for production of somatic embryos for artificial seeds (2), novel biotransformation (3), and expression of foreign proteins (4).

Unlike processing of microbial and animal cells in bioreactors, no economically viable process based on the suspension culture of plant cells in bioreactors has yet been possible in North America. This is owing largely to the cheap supply from traditional agriculture, and the small demand for most high-value products presently made from plants (5) and low productivity in plant cell culture. The major causes of low productivity of plant cell culture (about 0.02 g/L/d or less) are slow growth rate (<0.1 d^{-1}), low cell concentration (usually <10 g/L), and low product yield (typically <0.5 g/L) (*6*). It has been estimated that to sell products of plants at \$1000/kg based on their current market demands, the bioreactor productivity cost needs to be in the range of 0.12–0.15 \$/L/d (*5*). Clearly there is a need to increase the productivity of plant cell culture by 10- to 20-fold from its current level of not more than 0.02 g/L/d. This increase can be achieved by two approaches: (1) biological solutions, such as genetic transformation of cells to boost the expression of products, and stable hairy root cultures (*7*); and (2) engineering solutions, such as optimization of the medium and hydrodynamic environment of cells. This discussion concentrates on the second approach: process engineering.

Because plant cell culture must compete economically with agriculture, expensive complex technology must be avoided, and time-honored stirred-tank fermentation methods will have to be applied. For large-scale application of stirred-tank bioreactors for plant cell culture, effects of fluid forces on the growth and product expression levels have to be characterized, and mechanisms behind these effects have to be determined. The chief purpose of the study was to conduct experiments and develop a model to facilitate this fundamental understanding.

PLANT CELL CULTURE IN BIOREACTORS

Plant cells have been grown in a variety of bioreactors, such as roller bottles, rotating drums, stirred tanks, bubble columns, and air-lift columns

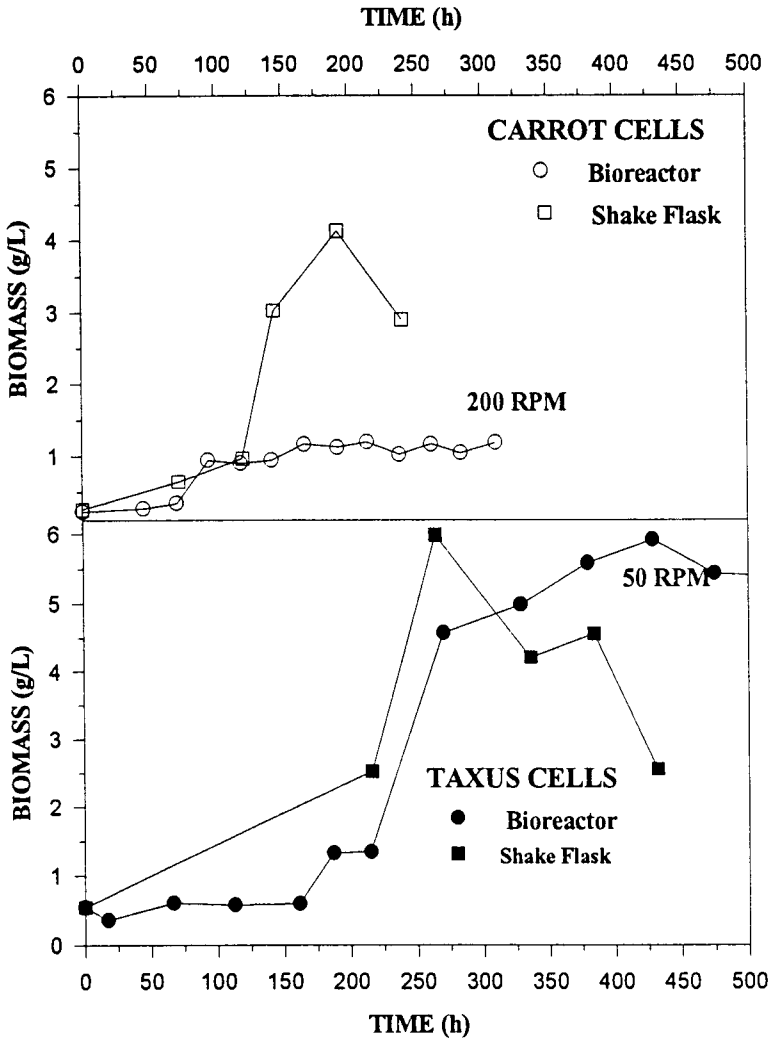


Fig. 1. Comparison of biomass production in shake flask and bioreactor cultures.

(*8*). Successes in producing biomass and metabolites in shake flasks and roller bottles, however, have been difficult to reproduce, let alone surpass, in a stirred bioreactor (*9,10*). One main reason for this difficulty is stated to be the hydrodynamic environment of the bioreactor, in which plant cells are sensitive to fluid forces and gas compositions (*11,12*). Suspension cultures of *Taxus brevifolia* cells for Taxol production were grown in our laboratory in a 3-L stirred bioreactor at different agitation speeds. Cells were grown successfully at 50 rpm to a maximum biomass level of 5.7 g/L, whereas no growth was observed at higher speeds of 100 and 150 rpm. This yield in the bioreactor was comparable to shake-flask yields, but it occurred a few days later than in shake flasks (Fig. 1). Suspension culture of *Daucus carota* (carrot) cells were also grown in the same bioreactor as used for *T. brevifolia*, but at the higher agitation speed of 200 rpm (Fig. 1). Carrot cells showed significant growth at this higher agitation speed, but it was much lower than that observed in the shake flask.

There are examples of plant cells grown successfully in "high shear" environments, possibly owing to the selection of shear-resistant strains through subculturing over a few years (*13*). Although some plant cell suspensions have been grown in stirred bioreactors up to 75,000-L scale (*14*), and grown to high cell density of 10–20 g/L, the shear sensitivity of other plant cells has led to the use of nonconventional impellers (*15*), and mild mixing and aeration conditions.

These conflicting reports on the plant cell cultures in bioreactors can be explained by determining the mechanism of fluid-mechanical sensitivity of plant cells. Extensive studies on the fluid-mechanical sensitivity of animal cells resulted in successful scale-up of freely suspended and microcarrier-attached cells in bioreactors (*16*). Cells are exposed to two types of fluid forces under typical agitation conditions in a stirred bioreactor. Close to the impeller, cells encounter time-varying turbulent forces caused by the fluctuating velocity components. In the bulk, however, cells are exposed to laminar shear forces associated with the gradients in the time-averaged flow. Turbulent forces have proven to be lethal for microcarrier-based animal culture. Also, high levels of shear stress for very short periods are produced by bubbles during bubble disengagement at the top liquid surface and bubble coalescence in the bulk (*17,18*). These bubble-based shear forces were found to be lethal for freely suspended animal cells, but not for microcarrier-based cell culture (*16*).

Based on this understanding of animal cells, we can assume that plant cell aggregates would be subjected to three forces in a bioreactor: agitation-based laminar forces, turbulent forces, and bubble-based forces. Large plant cell aggregates, from 100 μm to a few millimeters, would be relatively insensitive to bubble-based shear forces, as are microcarrier-based animal cells, owing to their larger size compared to the liquid film around bubbles. This insensitivity to bubbles has been demonstrated in a few studies (*19,20*). This article, therefore, concentrates on the effects of agitation-based fluid forces rather than on the lethal effects of bubble-based shear forces.

SUBLYTIC EFFECTS OF FLUID FORCES

Biological cells of all species encounter fluid forces in the microenvironment of their natural habitat as well as in artificial systems, and they usually produce a spectrum of adaptive metabolic and physical responses (*21,22*). Numerous nonlytic effects of fluid forces on animal cells have been characterized: increased product secretion (*23,24*), activation of intracellular signal transduction pathways (*25,26*), reorientation of the cytoskeleton (*27*), altered metabolic pathways (*26*), and opening and closing of ion channels (*28,29*), to name a few. These sublytic responses are in many cases characteristic to the tissue from which the cells were divided.

The performance of animal cells in suspension bioreactors, however, has been shown to be governed more by lysis effects (*16,30*) than by sublytic responses.

Microbial cells have a cell wall and therefore are assumed to be resistant to breakup, at least relative to animal cells. Contrary to expectation, a few reports show microbial cells to exhibit sublytic effects in highly turbulent bioreactors. Increasing levels of agitation have been shown to increase the sizes of *Escherichia coli* (*31*) and *Saccharomyces cerevisiae* (*32*) cells. The inhibition of growth, or "turbo-hypobiosis" effect, in microbial cells without any apparent physical damage has been also reported (*33*). Shear stress in a stirred bioreactor controlled the production of xanthan gum from a bacterial culture (*34*). Pressure-sensitive ion channels in *E. coli* have been also reported (*35*).

Unlike animal cells, but analogous to microbial cells, plant cells have a tough cell wall of tensile strength in the range of 1000–10,000 atm (*22,36*). Plant cells, like microbial cells, can be grown to a high cell density in bioreactors, up to 15 g/L (*37*). One would, therefore, expect plant cells to be shear-tolerant; however, the evidence that follows is contrary to this notion. Plant cells in several plant tissues are normally adapted to responding to mechanical forces. For example, a simple reorientation of columella cells of the root cap causes starch grains to change their intracellular distribution (*38,39*), the reorientation of a stem causes cell wall weakening (*40*), mechanical force causes pulvinar movement (*41*), and the bending of a stem (by wind, for example) causes increased cellulose deposition (*42*). All of these cellular responses are transient and lead to new steady states without irreversible damage to the cell. It is erroneous to believe that a cell that is resistant to fluid forces on the basis of cell lysis is unaffected by such fluid forces.

We have used various measures of "viability" to examine the sensitivity of carrot cells with the assumption that these measures should indicate the same effect as on growth and division (*43,44*). These measures of viability included regrowth ability, mitochondrial activity, membrane integrity, and lysis. The biological responses of cells in a bioreactor are assumed to be a cumulative effect of their exposure to different types of shear forces. If the high shear in the neighborhood of a cell, irrespective of the hydrodynamic environment (laminar, turbulent, or bubbles), is the main cause of the shear sensitivity, then the corresponding hydrodynamic variable needs to be identified. The viability data for carrot cells exposed to laminar forces in a Couette viscometer were correlated with the cumulative energy dissipation per unit volume (Fig. 2) on the cells during exposure to a particular flow condition. Based on this correlation, plant cells were observed to show a spectrum of fluid-mechanical sensitivity over six orders of magnitude of the cumulative energy dissipation (Fig. 3), from subtle effects on regrowth ability as indicated by colony forming ability and mitochondrial activity, to gross physical damage to membrane func-

From the Total K.E. Equation of Motion

$$\frac{\partial \bar{K}}{\partial t} + \bar{U}_j \frac{\partial \bar{K}}{\partial t} + \frac{1}{2}\left(\frac{\partial \bar{q}^2}{\partial t} + \bar{U}_j \frac{\partial \bar{q}^2}{\partial x_j}\right) + u_j \frac{\partial k'}{\partial x_j} =$$

Unsteady state terms

$$\left\{\frac{1}{\rho}\frac{\partial}{\partial x_j}(\bar{U}_i \bar{\tau}_{ij}) - \frac{1}{\rho}\bar{\tau}_{ij}\frac{\partial \bar{U}_i}{\partial x_j}\right\} + \left\{\frac{1}{\rho}\frac{\partial}{\partial x_j}u_i \tau'_{ij} - \frac{1}{\rho}\tau'_{ij}\frac{\partial u_i}{\partial x_j}\right\}$$

{Term A - Term B} + {Term C - Term D}

where,

$$\bar{K} = \frac{1}{2}\bar{U}_i\bar{U}_i \;,\; k' = \frac{1}{2}\left(2\bar{U}_i u_i + u_i u_i\right) \;,\; q = u_i u_i$$

$\bar{U}$ **= average velocity,** u_i **= fluctuations**

$\bar{\tau}$ **= shear stress,** τ' **= Reynolds stress**

Assuming Isotropic Turbulence locally for small eddies in the impeller region

$$\text{TERM C} = \text{TERM D} = \varepsilon_t$$

For a steady laminar flow in the Couette

$$\text{TERM A} = \text{TERM B} = \varepsilon_v$$

Assume the biological activity, B, of cells of volume fraction, φ, is exposed to a given flow condition of energy dissipation rate, $(\varepsilon_t + \varepsilon_v)$, for a period, t, then term B is given by

$$B = f\{E_c\} \quad \text{where,} \quad E_c = (\varepsilon_t + \varepsilon_v)\rho\varphi t$$

Fig. 2. Relationships for the concept of the total energy dissipation on the cells.

tion and cell lysis. Figure 4 also shows a stimulation in anthocyanin production by carrot cells while growth was inhibited. These important results in Figs. 3 and 4 show that the sublytic effect on growth and secondary metabolism, rather than physical damage or lysis, would control the productivity of plant cells in a bioreactor. Although Shuler (45) had speculated about the sublethal effects of shear in plant cell culture quite early, these are the first experimental results to suggest it.

A comparison of shear sensitivity of cells from various species using the cumulative energy dissipation on cells as a common basis was also performed (44). Figure 5 shows plant cells to have a similar degree of sensitivity on growth as insect and animal cells, but plant cells are resistant to membrane damage and lysis compared to animal cells, possibly because of their cell wall. Figure 5 also shows that any growth-inhibition effect of fluid shear in animal cells was apparently absent. To explain this difference in responses of animal and plant cells, plant protoplasts (cells without walls) were sheared under defined conditions. We observed that pro-

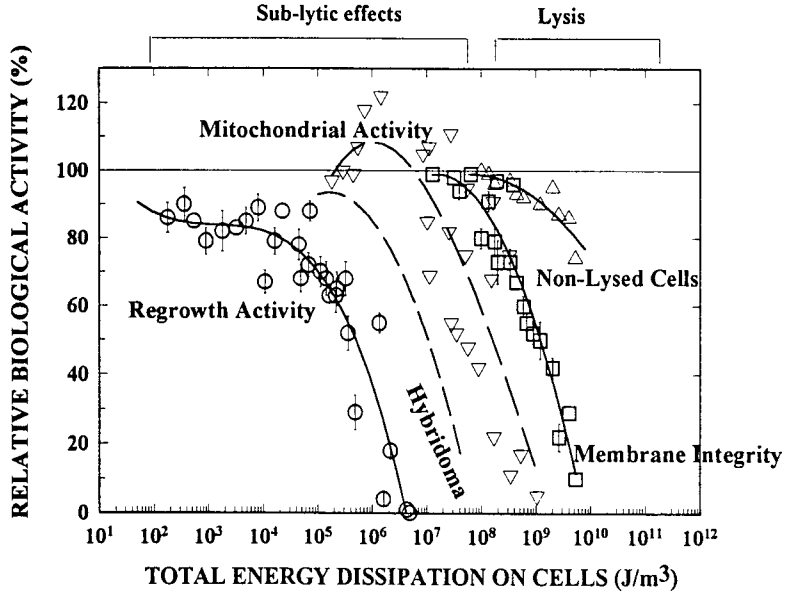


Fig. 3. Sublytic effects of fluid forces in plant cell culture.

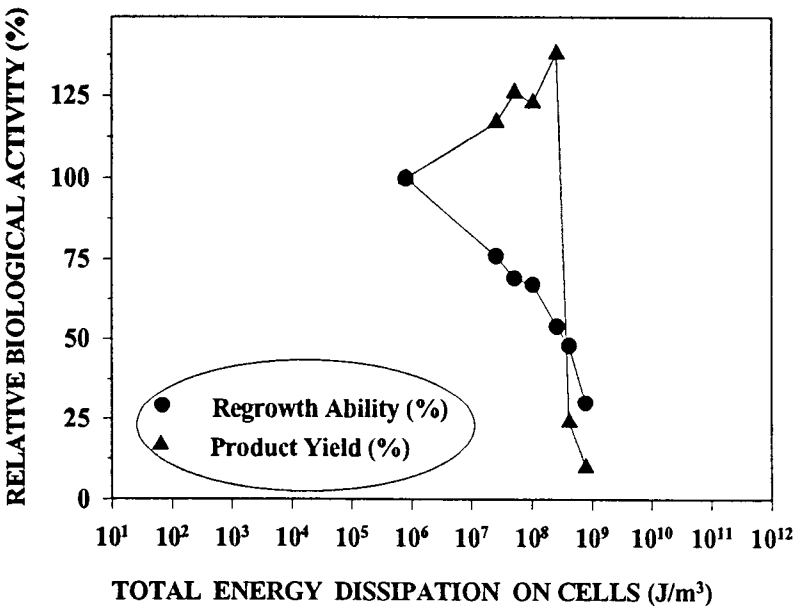


Fig. 4. Effect of shear on secondary metabolite production.

toplasts behave analogously to animal cells (Fig. 6) in that they do not show any separation in different viability assays. These results on protoplasts, plant cells, and animal cells show that plant cells can serve as a model to discern mechanisms of sublytic effects caused by fluid forces in the bioreactor.

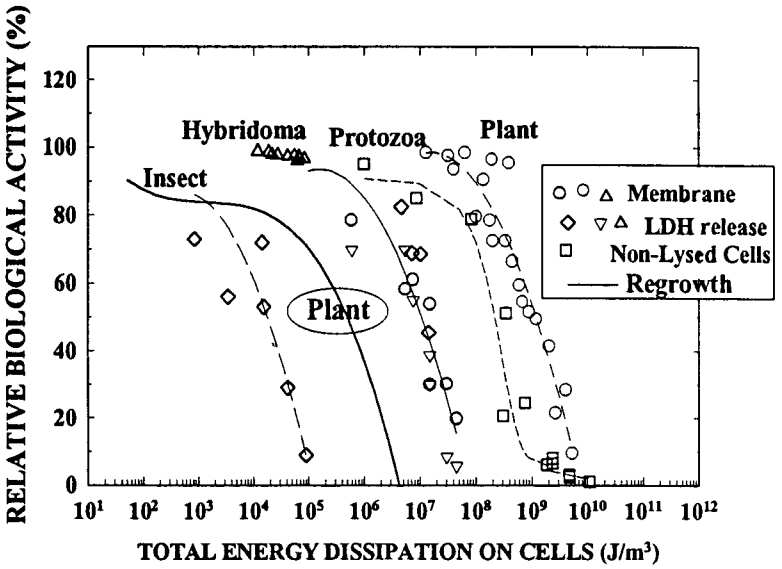


Fig. 5. Effects on functions of cells of various species under laminar forces.

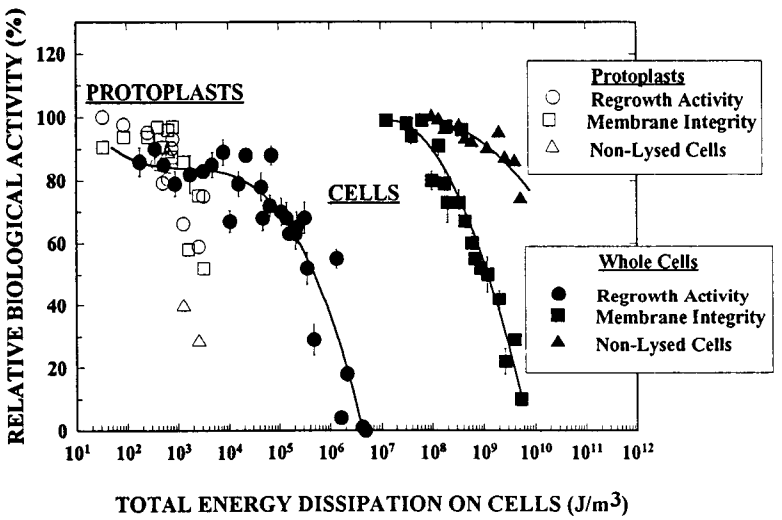


Fig. 6. Comparison of effects of laminar forces on plant cells and plant protoplasts.

THE MODEL

We have observed four critical physiological responses following the exposure of carrot cells to laminar shear in a viscometer for up to 1 h and over ten orders of magnitude in cumulative energy dissipation (Fig. 3) (43). We found that colony forming ability (regrowth), mitochondrial activity, and membrane integrity were affected at levels of energy dissipation on cells that were significantly lower than those that cause lysis of

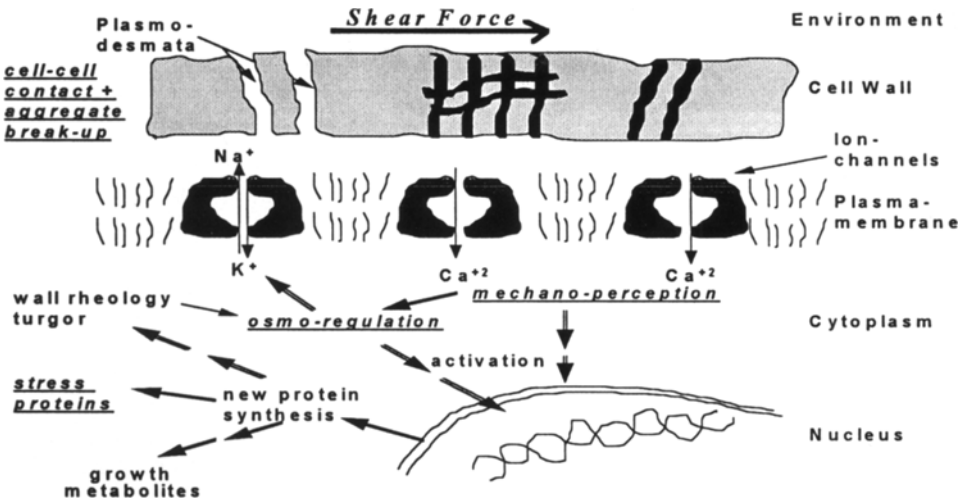


Fig. 7. Model for sublytic effects.

cells. Most alarming is the observation that plant cells loose their reproductive ability at about one-millionth of the energy required for lysis, making them approximately as sensitive to fluid shear as animal cells (note "hybridoma" curve in Fig. 3). Although many studies have shown that the lethal effects of fluid shear or lysis control the growth of animal cells in bioreactors (*16*), our results indicate that sublytic effects on growth are important in plant cell culture. We propose a tentative model in Fig. 7 for the sublytic effects of fluid shear on plant cells. This model governs our experimental plans. The mechanisms to be characterized are shown underlined. In the following sections, we will show how these mechanisms may play significant roles. This will lead to systematic design of experiments to evaluate roles of these mechanisms in producing sublytic effects.

Unique Properties of Plant Cells in Suspension

This model is based on certain unique properties of plant cells that are significantly different from those of microbial and animal cells (Fig. 8). These differences may explain why sublytic effects rather than physical damage or lysis would be an important factor for suspension culture of plant cells. These properties of plant cells, the medium osmolality, and hydrodynamic conditions have to be considered simultaneously in the designing of rational experiments to determine the mechanism(s) of the fluid-mechanical sensitivity of plant cells. We have selected four properties—calcium ion flux, stress protein expression, osmo-regulation, and cell-to-cell contact or aggregation, on the basis of their common role in stress responses in whole plants (22). A summary of these four properties

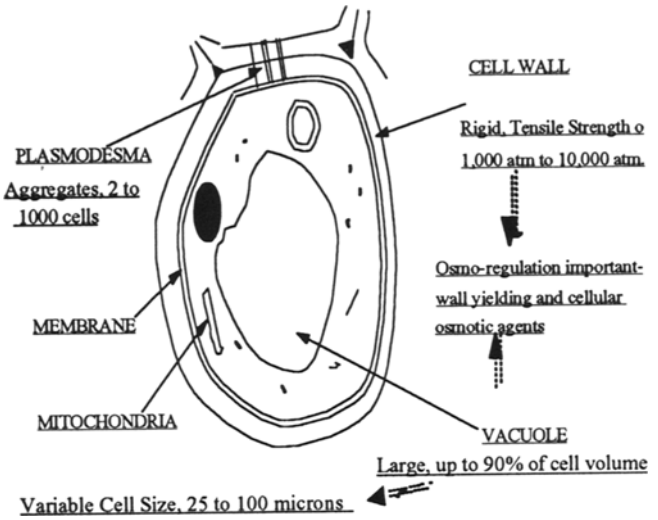


Fig. 8. Unique properties of plant cells.

is required to understand how they could play important roles in producing sublytic effects of fluid shear in plant cells.

Calcium Ion Flux and Mechano-Perception

Stretch-activated ion channels have been implicated in the transport of calcium ions in bacteria, yeast, animal, and plant cells (*35*). Yeh et al. (*46*) showed an increase in cytosolic Ca^{2+} level, $[Ca^{2+}]_c$, when insect cells were exposed to $1N/m^2$ of shear stress in a viscometer for 40 min. This condition corresponds to 2×10^4 J/m^3 of energy dissipation on cells, which is in the range 10^3–10^5 J/m^3 where growth inhibition of plant cells is observed in Fig. 3. The role of secondary messengers, such as $[Ca^{2+}]_c$, in modulating endothelial cell metabolism in response to fluid shear has been investigated (*47*). The $[Ca^{2+}]_c$ is tightly regulated in the nanomolar range since it affects a variety of physiological processes in plants, including cell wall material secretion, cell division, osmo-regulation, and membrane damage (*48*).

Responses of plants to physical forces in vivo have been studied recently in this context. For example, the effect of wind on whole plant growth (*49*) has been shown to be mediated through transient variation in $[Ca^{2+}]_c$ concentration. The fluctuating wind forces on tobacco seedlings immediately elicited a transient increase in $[Ca^{2+}]_c$ level, which increased as the applied force was varied from 6 to 12*N* (*49*). Osmo-regulation in plant cells, a metabolic activity essential for plant cell growth, has also been shown to accompany the increase in $[Ca^{2+}]_c$ levels (*50*). If the fluid shear force activates stretch-activated channels or affects turgor pressure (*see* later section on osmo-regulation), then $[Ca^{2+}]_c$ levels will be perturbed and will even-

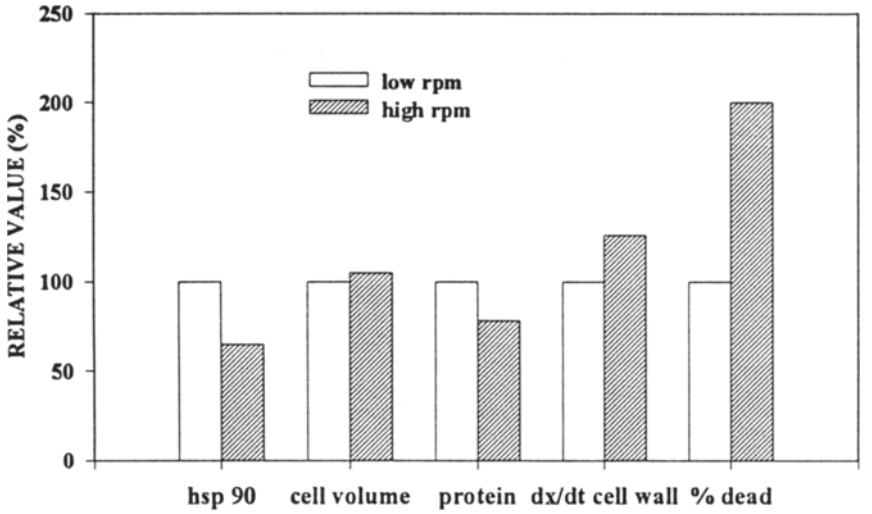


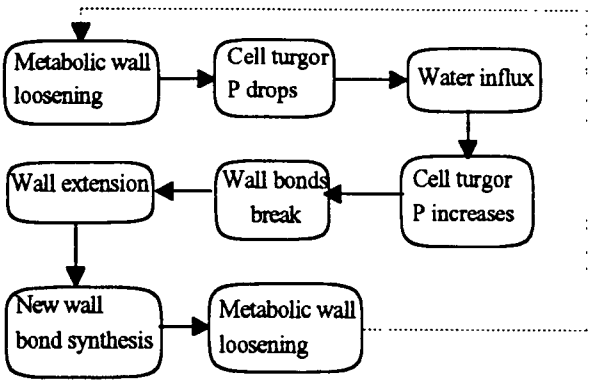
Fig. 9. Effect of agitation on yeast physiology in the bioreactor.

tually lead to sublytic effects on plant cells. These reports on plant cell physiology suggest that the sublytic effect on growth could be a result of cascade of events beginning with a transient change in $[Ca^{2+}]_c$ caused by fluid shear forces on cells in a suspension culture.

Stress Proteins and Stress Tolerance

Cells respond and sometimes adapt to a variety of environmental stresses by a rapid and transient acceleration of synthesis of certain proteins termed stress proteins (*51*). The elevation in expression of various sets of heat shock proteins (hsp) in response to heat stress, sometimes up to 1000-fold, is one of the best-characterized examples. These sets of hsp are also produced in response to a number of environmental stresses, including anoxia, glucose starvation, ethanol, arsenite, calcium ionophores, and viral infection. This stress response mechanism is conserved among a wide range of organisms, including *Escherichia coli, Saccharomyces cerevisiae,* plants, insects, and animals. Although the exact role of stress proteins is not clear, many studies indicate that they are involved in protection, recovery, and adaptation of cells to environmental stresses that denature intracellular enzymes.

If fluid forces in the bioreactor environment also elicit similar stress responses from cells as thermal stress, then sublytic effects on growth owing to fluid shear and gradual shear tolerance during successive subculture could be explained (*52*). A few studies with animal cells have shown no expression of any known stress proteins in response to laminar shear in a viscometer when intracellular protein synthesis was evaluated by 2D gel electrophoresis and ^{35}S-methionine labeling (*53,54*). In Fig. 9, experimental results from our laboratory show significant variation in the specific levels



$$\text{water influx} \quad \frac{1}{V}\frac{dV}{dt} = L(\Delta\Psi), \; where \; \Psi = P - \Pi$$

$$\text{cell growth} \quad \frac{1}{V}\frac{dV}{dt} = m(P - Y)$$

Fig. 10. Model for growth of a plant cell. Definitions given in text.

of a stress protein, hsp90, owing to step changes in the agitation speeds in the chemostat of yeast culture (*55*). Unlike previous studies (*54,55*), cells in this study were exposed to intense turbulent forces in the bioreactor, and an antibody specific to hsp90 was used to identify stress proteins. Concurrently, the wall composition (dx/dt) and fraction of viable cells were also affected, but no measurable change in steady-state biomass yield was observed. Investigation of the roles of stress proteins and $[Ca^{2+}]_c$ will help to identify a part of the cascade of events at the molecular level following the exposure of cells to fluid forces.

Osmo-Regulation and Fluid-Mechanical Sensitivity

Various environmental stresses, such as heat, freezing, salinity, drought, and water-logging are known to affect the growth of whole plants by interfering with osmo-regulation in cells (*56*). Osmo-regulation is an adaptive mechanism that alters the cellular osmotica to maintain the turgor pressue and to regulate cell wall composition. A quick look at the current model of cell growth (*57*) (Fig. 10) provides some clues concerning how osmo-regulation and alteration in cell wall composition could play a very important role in producing sublytic effects of fluid shear on plant cells and their dependency on age and species. Osmo-regulation of a cell depends on the strength of the cell wall, σ, cell turgor pressure, P, the cell wall extensibility, m, wall yield pressure, Y, and the ability to adjust the internal osmotic potential by transport of ions, or by synthesis or degradation of solutes. Cell volume enlargement (growth, dV/dt) is initiated only by irreversible metabolic yielding of the cell wall (*40*). Later, any changes in the osmotic potential of the medium, (Π_o) or elastic modulus of

the cell wall (ϵ) will induce water flux across the cell that is transient. Any stress, hydrodynamic or osmotic, can induce a variation in internal osmotic potential (Π_i) or elastic modulus of the cell, however, and interfere with cell growth.

Cell wall composition is the important link between fluid-mechanical sensitivity phenomena and osmo-regulation. Iraki et al. (*58*) have shown that the component of the cell wall that is responsible for its tensile strength is different from the component that is susceptible to metabolic loosening. Thus, a sublytic effect on growth could occur by affecting the cell wall composition without the wall becoming physically weak. Tanaka et al. (*19*) found that total wall content, and relative content of cellulose and hemicellulose increased under increasing turbulence in shake-flask and bioreactor experiments. The growth rate, however, was lower under the more turbulent conditions. Our studies on the effects of high turbulence on yeast physiology in a chemostat show significant variations in cell size, wall strength (Fig. 9), turgor pressure, and elastic modulus without significant changes in steady-state biomass yield (*53*). These studies demonstrate a strong possibility that interference with osmo-regulation and wall rheology can produce different sublytic effects.

Fluid-mechanical sensitivity of plant cells depends on cell age and type of species. For example, in actively growing culture, the osmotic gradient across the cell is the driving force for cell expansion and elongation. The cell wall can be fluid-like and extensible (large m and small Y) in growing cells or stiff and rigid (small m and large Y) in nongrowing cells. Hooker et al. (*59*) showed cells in late exponential phase to be more shear-sensitive than cells in lag and stationary phases, whereas Scragg et al. (*13*) found no effect of culture age. We have used sonication at noncavitation levels to apply short-term fluid stresses to carrot and taxus cells. Preliminary results in Fig. 11 show higher sensitivity of taxus cells compared to carrot cells in terms of mitochondrial activity as measured by red formazan product through the TCC assay (*60*). The study of the interaction of hydrodynamic stress with osmo-regulation mechanism, therefore, may explain the role of culture age and species.

A review of studies on fluid-mechanical sensitivity of any biological cells, including plant cells, reveals that the role of osmo-regulation was never considered. Shear studies with plant cells may explain a sublytic effect of fluid shear on biological cells in general in terms of osmo-regulation.

Aggregation of Cells

Plant cells in suspension usually exist as aggregates up to a few millimeters in diameter and containing up to hundreds of cells (*11*). The aggregate size distribution, which is usually a bimodal distribution, changes with different phase of growth and varies with species type (*61*). The aggregates are usually formed owing to incomplete separation of cells

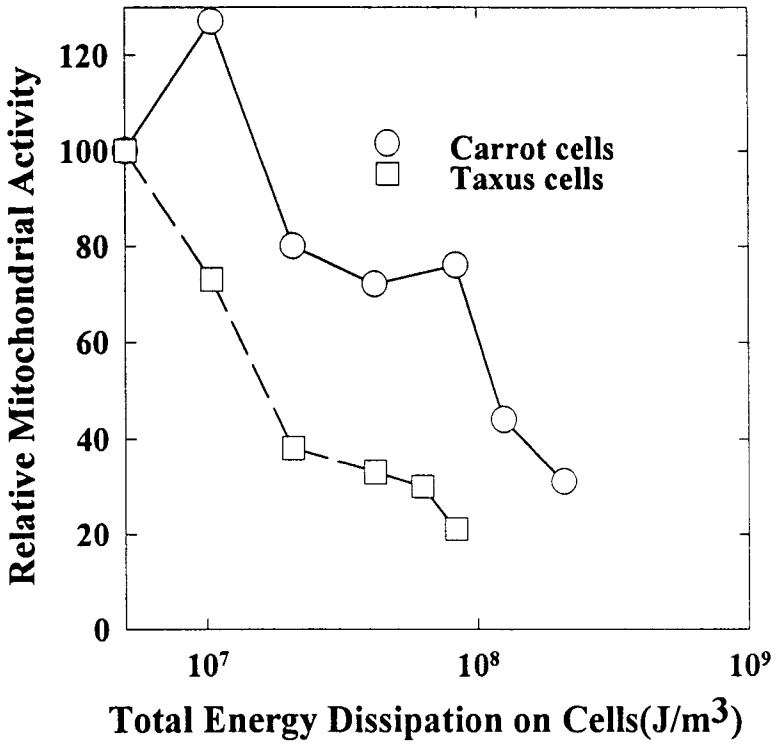


Fig. 11. Role of species in shear sensitivity of plant cells.

after division. Cells in aggregates are joined by the plasmodesmata through which metabolites of mol wt up to 1000 can be transported. During active growth, the aggregate size increases, but cells are smaller (*62,63*). During stationary phase, enlargement of cells on the surface of the aggregate is observed. Shuler (*45*) speculated that cells in aggregates are heterogeneous, and inner cells act as feeder cells to the cells on the edge. No study has yet been reported to suggest that plant cells can be grown continuously as single cells. Larger aggregates, however, need to be broken into smaller aggregates to avoid mass-transfer limitation and hollow centers owing to autolysis (*64*). For example, the mitotic index of smaller aggregates was found to be higher than that of larger aggregates (*65*).

The mode of breakup of aggregates may affect the viability of smaller aggregates if it affects the plasmodesmata contact or wall integrity. Aggregate size has also been shown to affect other metabolic activities, including the expressions of many enzymes, secondary metabolites, phenolic levels, and intracellular RNA, DNA, and protein levels (*11,66*). For example, the production of anthocyanin was increased by selecting smaller aggregates at every subculture for inoculation (*67*). These studies imply that plant cells in suspension exist as aggregates that are necessary for viability and product formation.

The reproduction of a desired aggregate size distribution is difficult at different scales from shake flasks to bioreactors (*68*). Various methods of mechanical, nutritional, enzymatic, or chemical strategies have been proposed in the literature to control size distributions for different purposes (*63,65,66,69*). In a bioreactor, the fluid shear will control the mode of breakage of aggregates. Scragg et al. (*13*) exposed plant cell aggregates to high turbulence in a 3-L stirred tank and measured aggregate size distribution and viability. They found that the original bimodal distribution became unimodal with the mean size reduced by a factor of four, but the growth remained unaffected. Our studies with carrot cells under turbulent forces show results opposite to this study (*43*). The aggregate size reduction occurred at a total energy dissipation four orders of magnitude greater than that required to affect the mitochondrial activity. A systematic approach is required to determine the relative contribution of different modes of breakage to overall size reduction by mechanical forces and its impact on viability and product formation.

PREVIOUS EXPERIMENTAL APPROACHES

Previous studies have correlated the operating conditions of bioreactors with biological responses, including lysis, biomass weight, cell size distribution, and product formation (*10,13,14,59,70*). A major drawback in these studies is that the cells experience various types and levels of fluid forces in the bioreactor. A few studies have been done to evaluate systematically the fluid-mechanical sensitivity of plant cells in controlled shear environments for a short duration (*13,59,71,72*). *Catharanthus roseus* cell culture was exposed to turbulent forces for short and long periods in stirred bioreactors (*13,71,72*). No effect on growth of *C. roseus* cells was observed, but aggregate breakup occurred. Tobacco cells were sheared in a coaxial cylinder system at the maximum shear stress of $1.3 N/m^2$ for up to 6 h (*59*). A decrease in mitochondrial activity and an increase in lysed fraction of tobacco cells were observed as the shear rate and exposure time were increased. These two studies show conflicting results on the sensitivity of plant cells to agitation-based shear forces. It cannot be determined whether this difference in the sensitivity of plant cells is the result of viability, differing species, culture history, or different flow environments.

The productivity of a plant cell culture in a bioreactor would depend on the ability of cells to grow and divide, or "viability," and to produce a desired metabolite. In all except the experiments by Scragg et al. (*13*), viability was estimated by indirect measures, such as mitochondrial activity, membrane integrity, or lysis. These biological responses represent physical damage to cells by fluid forces in the lethal range. Also, these studies did not evaluate the effects of fluid forces on product formation

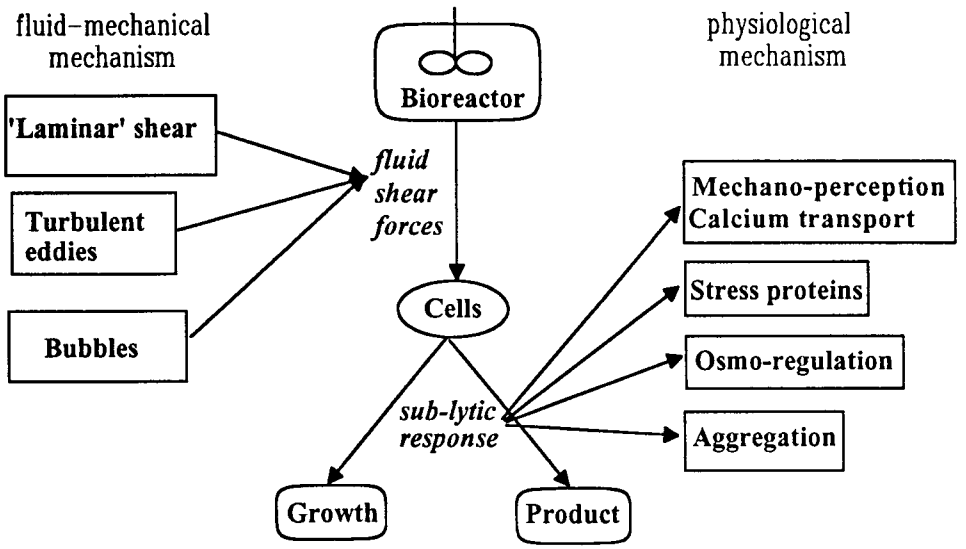


Fig. 12. A framework for future studies.

per se. For example, many plant cell cultures show induction of secondary metabolite production in the presence of environmental stresses, such as fungal extracts, light, and oxygen limitation (*73*). A recent literature survey on animal cells (*18*) and our preliminary results on plant cells show that a number of nonlethal or sublytic responses are possible under modest agitation conditions that will affect growth and product formation. Neither the nonlethal forces causing these sublytic responses nor the effects of these sublytic responses on productivity of plant cell culture have been studied so far. The understanding of the critical fluid forces and the mechanisms of the sublytic responses they produce is essential for creating rational strategies of plant cell bioreactor operation. This new approach to shear sensitivity of plant cells is presented in Fig. 12. This requires designing systematic experiments to determine roles of these four unique properties of plant cells as described in the model (Fig. 7).

FUTURE DIRECTIONS

The study of sublytic responses of plant cells to fluid forces has two main components (Fig. 12): the physiological (or cellular) mechanisms underlying the observed response and the identification of fluid-mechanical (or hydrodynamic) mechanism for the cause. Once the dominant cellular and hydrodynamic mechanisms are identified, biological and engineering solutions to the problems of fluid-mechanical sensitivity of plant cells to fluid forces can be determined. The main objective of any future systematic study, therefore, should be to identify dominant mechanisms causing sublytic effects in plant cells in bioreactors at physiological

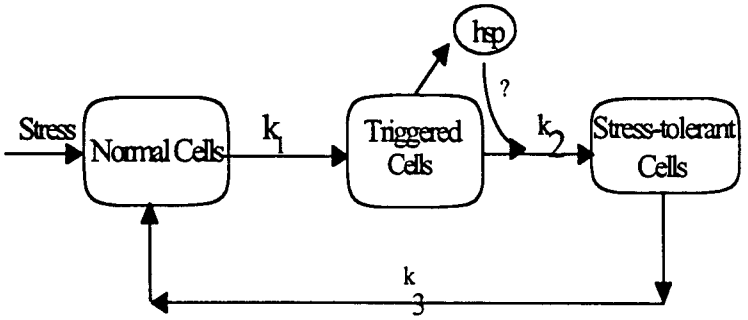


Fig. 13. Model of stress response and tolerance.

and fluid-mechanical levels. We propose four specific goals to achieve this objective. Each is designed to identify a physiological/cellular mechanism by applying an appropriate fluid-mechanical stimulus. These goals are identified below.

Mechano-Perception of Fluid Forces and Its Impact on Growth and Metabolism

The hypothesis is that plant cell responses to hydrodynamic forces involve a biological mechanism of perception and an active physiological response. Hydrodynamic forces cause transient changes in cytosolic calcium, $[Ca^{2+}]_c$ (*74*), that initiate a cascade of events leading to sublytic effects on growth and product formation.

Role of Stress Proteins in Sublytic Effects on Plant Cells

The hypotheses are as follows: (1) Fluid shear stress induces the expression of certain stress proteins and inhibits/represses synthesis of certain other proteins. (2) These stress proteins play a role in helping cells to adapt to fluid stress environments. The role of hsp, which are usually expressed under a variety of environmental stresses and may act as per the model in Fig. 13 (*75*), will affect the growth of plant cells exposed to fluid forces.

Role of Osmo-Regulation in Sublytic Effects on Plant Cells

The hypotheses are as follows: (1) Fluid forces interfere with osmoregulation and therefore produce sublytic effects (Fig. 14). (2) Turgor and wall properties of cells can be modified by adapting cells to higher levels of osmotic stress than that of usual medium, thereby resulting in a different degree of sensitivity to fluid stress (Fig. 15). It can be determined whether the sublytic effect of fluid forces on growth and secondary metabolite production is mediated through interference with the osmo-regulation ability of cells and cell wall composition (*76,77*).

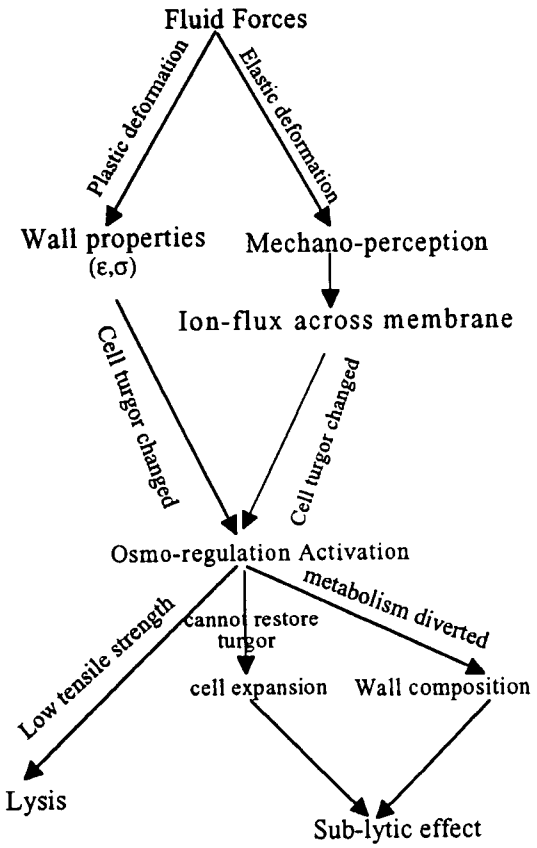


Fig. 14. Effect of fluid forces on osmo-regulation.

Mechanism of Aggregate Breakup and Its Impact on Growth

The hypothesis is that the mode of separation of cells from aggregates and aggregate breakup (Fig. 16) (*78*) will affect cell viability and the expression of product. The relevance of mode of aggregate breakup and cell-to-cell contact to growth and secondary metabolite production should be evaluated.

CONCLUSIONS

Successful cultivation of plant cell culture in large bioreactors is one of the critical technical bottlenecks to be overcome for realizing full potential of plant cells. The least expensive approach to selection of suitable strain and cell culture method will be required to make this approach profitable, and this implies using stirred-tank fermenters and confronting the

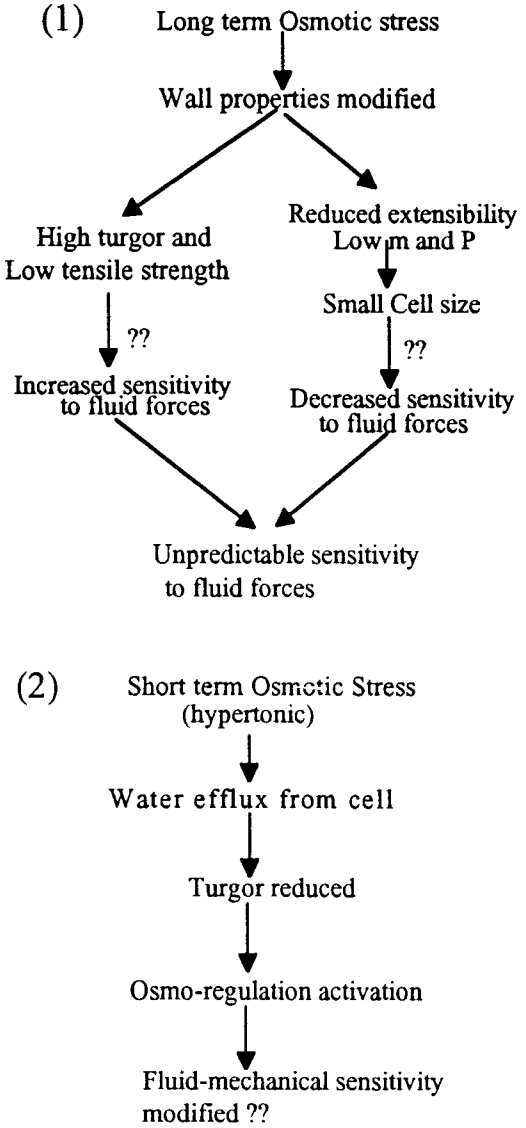


Fig. 15. Role of osmotic stress in shear sensitivity.

associated shear fields. Our recent research demonstrated that many unknowns about fluid-mechanical sensitivity of plant cells persist. It will be necessary to reveal the facts about the sublytic responses of plant cells to fluid shear. Specifically, a general mechanism based on sublytic responses, ranging from mechano-perception to inhibition or stimulation of growth/product through stress proteins, osmo-regulation, and aggregate breakup, is needed. Also, the magnitudes and types of fluid forces required to produce these effects must be determined.

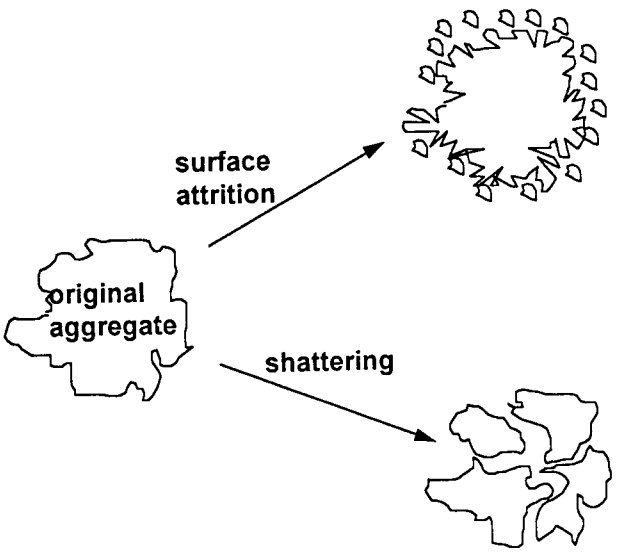


Fig. 16. Modes of aggregate breakup.

On the basic research side, suitable design of experiments will lead to explanations of the role of sublytic effects in the culture of cells in general, and will develop a general fluid-mechanical and physiological mechanisms for interaction of fluid forces with biological cells. On the very practical side, the proposed responses can be used for screening plant cell strains for their suitability for cultivation in stirred-tank reactors. For example, a laminar-shear device and a turbulent-shear device could be used to stress candidate cell lines whose tolerance could be tested by a stress-protein assay, aggregate size measurements coupled with survival assay, and survival tests under various osmotic pressures. These tests in bench-scale equipment will pave the way to determining engineering and genetic/physiological solutions in a rational fashion. These solutions will facilitate the transition to commercial plant product synthesis in suspension bioreactors and selection of strains suitable for large-scale production.

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