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Photobioreactor Culture of Photosynthetic Soybean Cells

Growth and Biomass Characteristics

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ABSTRACT

Photosynthetic suspension cultures of higher plants offer an alternative approach to biomass production, potentially yielding cellulosic material and protein on a continuous year-round basis. A bench-top hybrid photobioreactor was developed to study photomixotrophic and photoautotrophic growth of *Glycine max* as a model system. Maximum biomass doubling times for photomixotrophic and photoautotrophic growth were 1.87 and 3.92 d, respectively. The presence of exogenous sugars resulted in photomixotrophic growth, reduced chlorophyll levels, and a reduction in photosynthetically-evolved oxygen. Depletion of carbohydrates from the medium coincided with the beginning of stationary phase and an increase in oxygen evolution by the cells. A second growth phase, prolonging cell viability, could be initiated by increasing the carbon dioxide from 2 to 5%, just before the onset of stationary phase. Biomass from bioreactor cultured cells proved resistant to enzymatic attack without pretreatment. Composition of the biomass was 7.8% lignin, 20.7% α-cellulose, 23% hemicellulose, 5.5% starch, 14.5% protein and 6.5% nucleic acids.

Index Entries: *Glycine max;* photobioreactor; suspension; photomixotrophic; photoautotrophic; biomass.

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INTRODUCTION

Since the late 70s, one approach suggested for extending our liquid engine fuel supplies has been through alcohol derived from renewable resources. Typically, agricultural crops would be grown for starch or cellulose with subsequent hydrolysis and fermentation, or forestry and agricultural residues used as feeds for gasification/methanol synthesis. An alternative approach could be the scale-up of photosynthetic higher plant suspensions in large, sun-driven bioreactors coupled to CO_2 generating plants, such as breweries or coal fired power plants.

This system would have several advantages over current methods

1. Plant cell lines could be developed with desired properties.
2. Consistent biomass would allow optimization of pretreatment and hydrolysis procedures with minimal changes between runs.
3. The cost of transportation would be eliminated owing to on-site conversion.
4. More biomass could be produced per hectare than field grown crops.
5. In view of global climatic changes and severe water shortages in many areas of the world, bioreactor culture of biomass could provide a means of utilizing waste CO_2 while recycling valuable water.
6. Finally, biomass production would be year-round and not seasonal.

Photobioreactors require a large surface area for illumination, reactor walls to be constructed of transparent material, and a relatively thin liquid level for maximum light penetration, factors that limit bioreactor size. Modification of existing bioreactors would greatly improve the economics and acceptance by industry. Plant suspensions, cultured in bioreactors, require conditions different from traditional microbial fermentations, such as high light intensities, both CO_2 and O_2 and are more sensitive to shear owing to cell size and a rigid cellulose cell wall (*1*). For further information on plant bioreactor systems and scale-up principles, *see* reviews by Martin (*2*) and Spier and Fowler (*3*).

Photomixotrophic (PMT) and photoautotrophic (PAT) suspensions of higher plants are increasingly employed to study metabolism, but culturing cells for biomass and subsequent conversion to fuels and chemicals has been neglected. The purpose of this research was to determine the composition of biomass produced from PMT and PAT suspensions in photobioreactors and its enzymatic digestibility. It is hoped that this effort will stimulate further interest in this area.

METHODS

Cultures

The soybean PMT suspension culture SB-P (*Glycine max* L. Merr. var. Corsoy) (*4*) was originally obtained two years ago from S. M. D. Rogers of the Texas A & M University Horticulture Department, College Station, Texas. The SB-P cell line has been subcultured in our laboratory on KN1 medium (*5*) every 14 d (late exponential phase) using an inoculum ratio of 1:8 (10 mL inoculum to 80 mL medium in 250 mL nonbaffled flasks sealed with parafilm) and maintained under constant fluorescent cool white illumination (250–300 $\mu E/m^2\ s^1$) at 27°C on an orbital shaker (120 rpm, 2.54 cm stroke). A shear tolerant cell line was developed in 250 mL four baffle flasks, subcultured as the SB-P line under constant illumination (200–240 $\mu E/m^2\ s^1$) at 27°C on an orbital shaker (200 rpm, 2.54 cm stroke). A PAT cell line SB-A) of soybean, also provided by S. M. D. Rogers, was compared with the PMT cell line (SB-P).

Stirred Tank Bioreactor

Several New Brunswick Bio-Flow II (New Brunswick Sci. Co., New Brunswick, NJ) stirred tank reactors (STR) with a 1.25 L working volume was modified to photobioreactors, as previously described (*6*). Agitation was provided by a flat bladed turbine (5.0 cm diameter), cell lift impeller, or marine impeller (5.0 cm diameter). The flat bladed turbine and marine impeller were also utilized in pairs. The ring sparger was replaced with a ceramic stone that reduced bubble sizes to 1–3 mm and tripled the volumetric oxygen transfer coefficient (K_La) values. Air flow rate and composition were measured with flowmeters and expressed as aeration rate in volume of air per volume of medium per minute (vvm). PMT and PAT gas composition was 2 and 5% CO_2, respectively, in a carrier gas composed of air. The inoculum was from 14- to 16-d-old shake flasks using a ratio of inoculum to reactor volume of 1:8. Reactor conditions were constant illumination (180–200 $\mu E/m^2\ s^1$ measured at inside glass surface), 26°C and an initial pH of 5.4, with no attempt to control pH. Impeller speeds (30, 60, 100, and 200 rpm) and vvm (0.1, 0.2, 0.3, 0.4, and 0.5) were varied, depending on impeller configuration, to produce best mixing without excessive foaming. Dissolved oxygen (DO) was monitored, but not maintained at a preset level. Data to include pH, redox, temperature, and DO were continuously monitored and logged by the Loggernaut Data Logging System (Cyborg Corporation, Newton, MA).

Biomass

Fresh and dry cell weights were determined using a filtration method. Each sample of 100 mL, representing 9.0% of the total bioreactor volume,

was divided into 2×50 mL aliquots. Fresh weights were determined by filtering cells through a preweighed wet filter, washing the filter and cells once with distilled water, and weighing. For dry weight measurements, cells were filtered through preweighed dried (105°C for 24 h) filters, washed once with distilled water, then dried at 105°C for 24 h and weighed. Fresh and dry weight data points represent one sample from each of three bioreactor runs.

Cell Viability

Cell viability was determined by staining dead cells with phenosafranine and live cells with fluorescein diacetate, according to the method of Widholm (*7*).

Particle Size Determination

The size distribution of cell aggregates in the bioreactor and shake flasks was determined by washing 100 mL through a range of pretared polypropylene Spectra/Mesh Filters (Medical Industries, Inc., Los Angeles, CA) with pore sizes of 1000, 500, 350, 210, and 105 μm. After fresh weight measurements, cells were washed from filters and deionized water into pretared glass vessels, dried overnight at 105°C, and weighed. Microscopic analysis was also used to determine whether cell debris or ruptured cells were present.

Chlorophyll Determination

Chlorophyll (Chl) was extracted with ice cold acetone/water mixture (80/20, v/v) from cells (250–750 mg fresh weight) and total Chl calculated from Chl a and b, according to the method of Hipkins and Baker (*8*), and expressed as μg Chl per gram fresh weight (GFW).

Carbohydrate Determination

Sucrose, glucose, and fructose concentrations in the medium were determined as previously described (*6*) Starch was determined according to Cobb (*9*) and reported as a percent of cell dry weight. Callose was determined by the staining method of Kohn et al. (*10*).

Enzyme Digestions

Enzyme digestions used 50 mg air dried sample and 1.3 U (26 filter paper U/g sample) Genencor GC 123 cellulase preparation in 5 mL (total vol) 50 m*M* sodium acetate buffer, pH 4.8. Enzyme digestions of white fibrous extracellular material used 5 mg air dried sample and 0.13 U enzyme preparation (26 FPU/g sample) in 2 mL buffer (total vol). All enzyme digestions were carried out for 72 h at 50°C and were then placed in a 70°C bath for 20 min to precipitate the enzymes. Sigmacell Type 50

(Sigma Chemical Co., St. Louis, MO) was used as an external control to indicate hydrolysis of 95% or better after 72 h.

Pretreatment consisted of swelling air dried material in KOH (1 and 5%) for 12 h using 1 mL KOH solution to 8 mg sample. Acid hydrolysis used 3% H_2SO_4 for 3 h at 50°C. Total reducing sugars after enzymatic and acid hydrolysis were measured as free glucose equivalents using the DNS method (*11*).

Cell Wall Composition

Lignin was determined as 72% H_2SO_4 acid-insoluble Klason Lignin (*12*). Holocellulose was determined by the chlorite-acetic acid method, which solubilizes lignin leaving holocellulose (*13*), then fractionated with 18% KOH to α-cellulose and hemicellulose (*14*). No attempt was made to quantitiate glucomannans and xylans.

Photosynthetic Potential Estimations

The rate of photosynthetic evolution of oxygen was measured using a YSI Model 5300 Biological Oxygen Monitor and variable speed water bath assembly (Yellow Springs Instruments Co., Inc., Yellow Springs OH), as previously described (*6*).

Statistical Analysis

The statistical software package used to analyze the raw data was StatView 512+ (Abacus Concepts, San Diego, CA). The mean and the standard deviation were calculated from three experiments containing three samples for all values. Standard deviation values were included in figures and tables only when the standard deviation exceeded 5% of the mean.

RESULTS AND DISCUSSION

Figure 1 shows typical PMT growth of soybean resultilng in biomass doubling times (Td) of 1.87 d in medium containing 1% sucrose and a gas composition of 2% CO_2 in bulk air. Owing to the competitive binding of oxygen and carbon dioxide by ribulose 1,5-bis-phosphate carboxylase/oxygenase (EC 4.1.1.39, Rubisco), the gas phase becomes critical in determining whether conditions are favorable for CO_2 fixation by PMT tissues. Previous studies by Treat et al. (*6*) with 2% CO_2, 15% O_2 in bulk N_2 resulted in specific growth rates of $\mu = 0.44$/d and Td = 1.57 d. Reduced oxygen tension was used in an attempt to increase net photosynthesis by reducing competitive binding of oxygen by Rubisco. The economics of scale-up may prohibit a lowering of the oxygen tension, increasing the

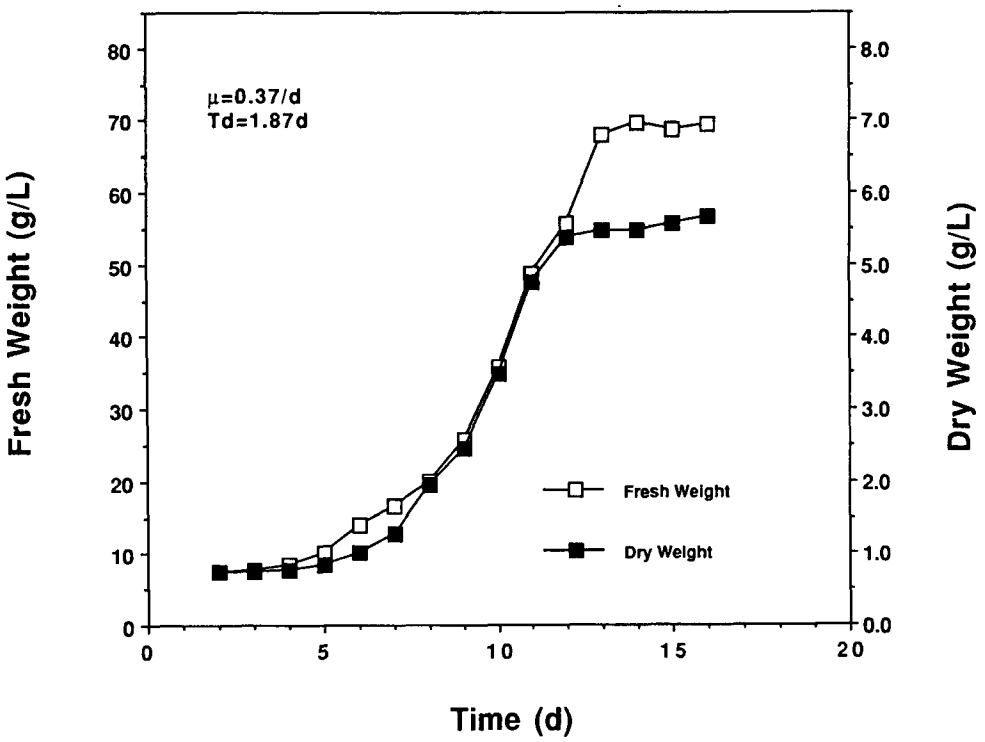

Fig. 1. Photomixotrophic growth of soybean in a 1.25 L bioreactor.

need to develop cell lines capable of photosynthetic growth at atmospheric O_2 levels.

Figure 2 shows a biphasic growth curve observed when the gas composition was changed from 2 to 5% CO_2 at the onset of the stationary phase. Carbohydrate analysis indicated that by d 10–12, sucrose was exhausted from the medium and little glucose and fructose remained. The composition of the gas phase appears to be critical. Data suggests that increasing the CO_2 composition of the gas phase to 5% at the time the cells enter the stationary phase has some yet unknown stabilizing effect to the cells. Spilatro and Anderson (*15*) observed that, upon exhaustion of exogenous sugars from the medium, *Glycine max* exhibited increased photosynthetic oxygen evolution and increased accumulation of Chl, both characteristics of PAT growth. During the exponential phase, in the presence of exogenous sugars, cells exhibited PMT growth. These observations suggest that additional biomass can be obtained by manipulation of the gas phase. Although CO_2 is important, oxygen must also be provided for proper growth, even for photoautotrophs. Depending on whether exogenous sugars, such as sucrose, are present, photosynthetic cell lines have been shown to shift between PAT and PMT growth (*16,17*).

Dissolved CO_2 has been suggested to be involved in cell permeability and is necessary for initiation of growth (*18*). Comparing PMT vs biphasic growth by viability staining showed contrasting cell conditions. PMT cul-

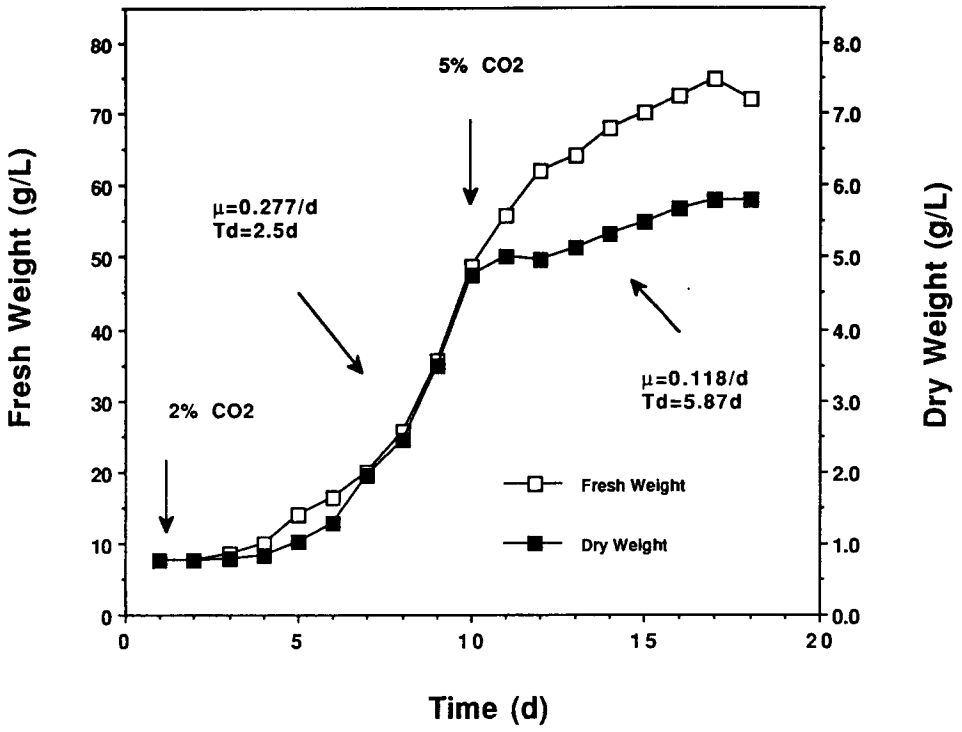

Fig. 2. Biophasic growth of soybean with photomixotrophic (Td=2.5 d) and photoautotrophic (Td=5.87 d) and step increases in carbon dioxide.

tures showed a rapid loss in cell viabilty when cells entered the stationary phase. When CO_2 levels were elevated, as cells enter the stationary phase, a greater than 90% viability was observed after 4 d. This compares to a reduction in viability to 65% after 3 d of stationary phase for PMT cultures. Although Td was 5.87 d after the addition of 5% CO_2, cell senescence began within 48 h without a step increase in CO_2.

PAT growth, shown in Fig. 3, differed from PMT growth by exhibiting a shorter lag phase, but longer biomass doubling times (3.92 d). Interestingly, PAT growth did not result in the rapid drop-off in Chl levels observed during lag and early exponential phase in PMT growth. Chl levels (μg/GFW) in early exponential phase were 475 (PMT) and 1065 (PAT), whereas late exponential phase contained 610 (PMT) and 1175 (PAT).

Table 1 shows maximum specific growth rate (μ) and biomass doubling time (Td) for shake flask and bioreactor cultures. PMT growth (Fig. 1) on KN1 medium (1% sucrose), with a gas composition of 2% CO_2 in bulk compressed air, resulted in Td=1.87 d and μ=0.37/d. Dry weight as a percentage of fresh weight varied from 7 to 10% in the exponential phase and 5 to 7% in the stationary phase. Photosynthetic oxygen potential, used to estimate the photosynthetic capabilities of green tissues, was 60–65 μmol O_2/h mg Chl for PMT and 95–105 μmol O_2 /h mg Chl for PAT in the midexponential phase.

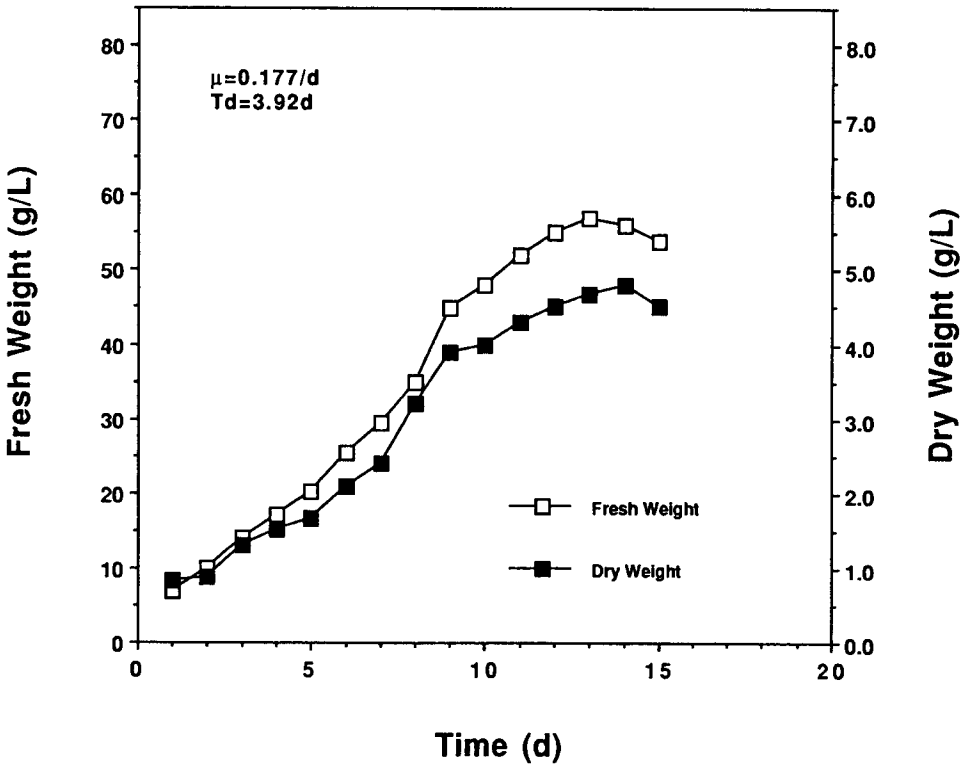

Fig. 3. Photoautotrophic growth of soybean.

Table 1
Growth Parameters for Soybean Culture[a]

Culture conditions	Specific growth rate, μ	Doubling time, d
Shake flask (120 rpm), photomixotrophic	0.176	3.94
Shake flask (200 rpm), photomixotrophic	0.206	3.36
Shake flask (120 rpm), autotrophic	0.156	4.44
Bioreactor, photomixotrophic	0.371	1.87
Bioreactor, biphasic	0.277	2.50
Bioreactor, autotrophic	0.177	3.92

[a]Td and μ based on dry weight.

Maximum cell density, on a dry weight basis, approached 6 g/L (Figs. 1–3). Dry cell densities up to 20 g/L dry wt have been achieved for non-photosynthetic higher plant suspensions in bioreactors (*19*). Lower densities observed for photosynthetic soybean may be a function of reduced light penetration at higher cell densities, depletion of a critical nutrient, nonoptimized gas composition or mass transfer limitations, or unrecognized physical contraints of the bioreactor. Wilson (*20*) found that, at cell densities above 20 g dry wt/L in an air-lift reactor, the air bubbles did not provide satisfactory mixing with the static regions forming, suggesting that this type of reactor is not suitable for culturing plant cells at high cell densities.

Deposition of cells on the reactor walls above the fluid level many times exceeded 30% of the total biomass and presents a significant problem with bioreactor culture of plant cells. Deposition of cells became especially troublesome as cells approached the stationary phase, when the cell volume expanded, but dry mass remained constant in a manner similar to the stationary phase observed in callus (*21*). Some separation of cells from the medium resulted from slight foaming and natural cell stickiness from increased extracellular polysaccharides (ECP).

Figure 4 shows typical carbohydrate utilization by PMT suspensions and an overlay of dry biomass accumulation. During the lag phase (0–3 d), a rapid extracellular conversion of sucrose to glucose and fructose occurred. This conversion of sucrose to glucose and fructose was probably owing to the extracellular and cell wall bound invertase detected in the culture filtrate and cell wall debris. Stanzel et al. (*22*) showed that sugar concentrations about 1 m*M* exhibited linear, diffusion-like uptake for *Streptanthus* suspensions. During exponential growth, a preferential use of glucose over fructose was observed. Carbohydrate uptake studies by Stanzel et al. (*23*) also showed a preferential uptake of glucose over fructose. During lag and early exponential phase (dotted line, Fig. 4), when the exogenous carbohydrate level was high, cellular Chl (475 ± 38 μg/GFW) levels decreased, and cells showed a marked decrease in their ability to evolve oxygen (32 ± 6 μmol/h mg Chl). The Chl (610 ± 48 μg/GFW) level and the ability of cells to produce oxygen (75 ± 7 μmol/h mg Chl) increased in the late exponential and stationary phase, concomitant with the onset of PAT growth.

Figure 5 shows net biomass yield after 14 d of culture as a function of impeller type and impeller rpm. Highest biomass yields were obtained with a pair of 5 cm marine impellers and the cell-lift impeller, both of which fill within overlapping standard deviation bars. A rapid reduction in biomass yield was observed with a pair of 5 cm flat blade impellers as the rpm was increased beyond 60 rpm. In culturing photosynthetic plant cells in bioreactors, it is necessary to maintain optimum mixing and aeration without increasing they hydrodynamic and mechanical stress effects beyond tolerance levels. Tolerance levels must be established for each cell

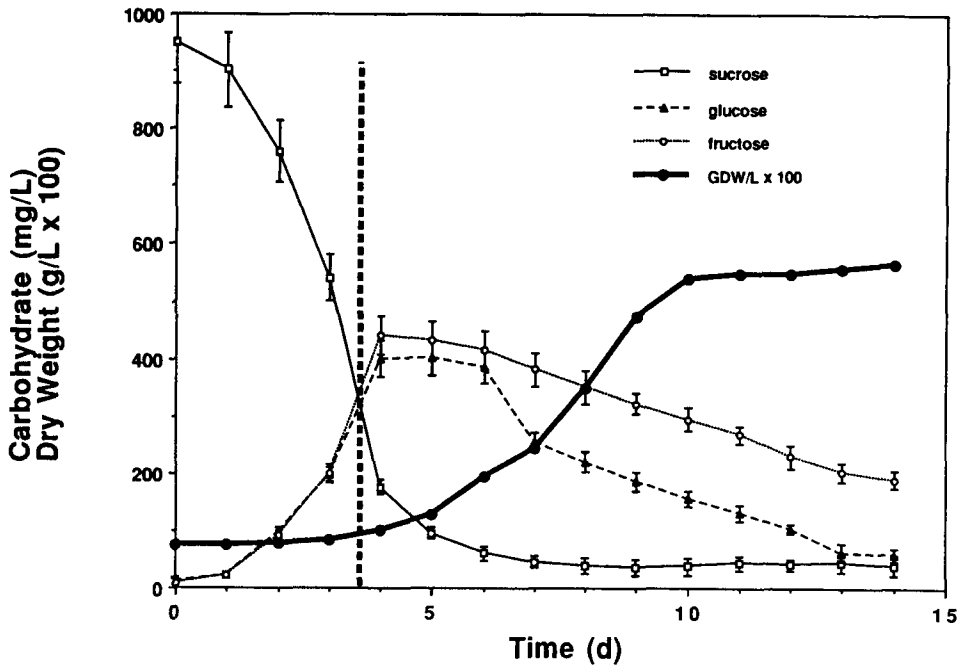

Fig. 4. Changes in the carbohydrate composition of the medium with dry weight biomass changes superimposed. Dotted line indicates beginning of the exponential phase.

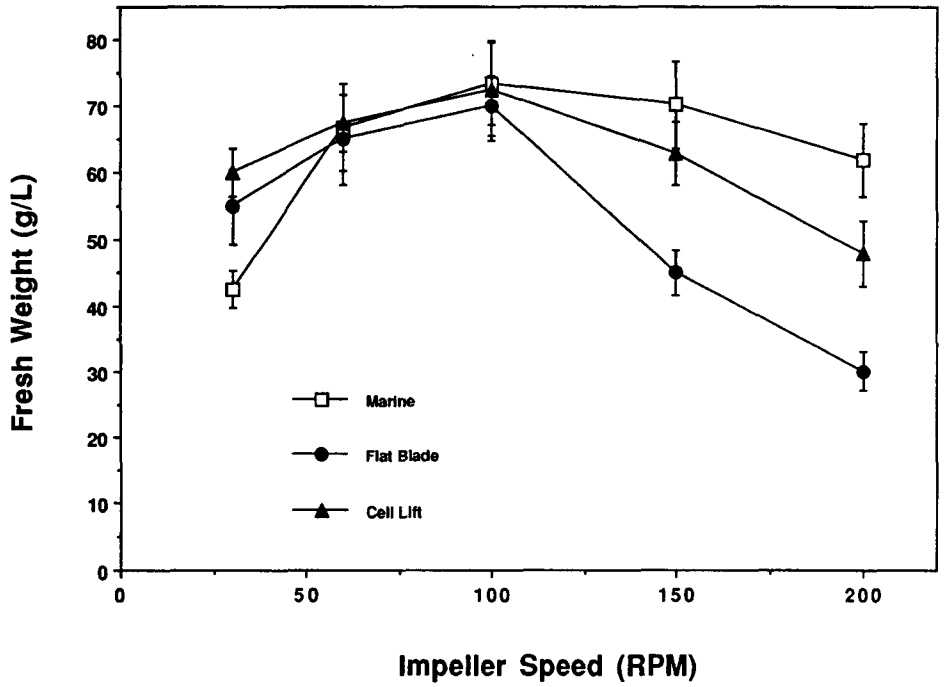

Fig. 5. Biomass accumulation after 14 d of culture as a function of impeller configuration and speed.

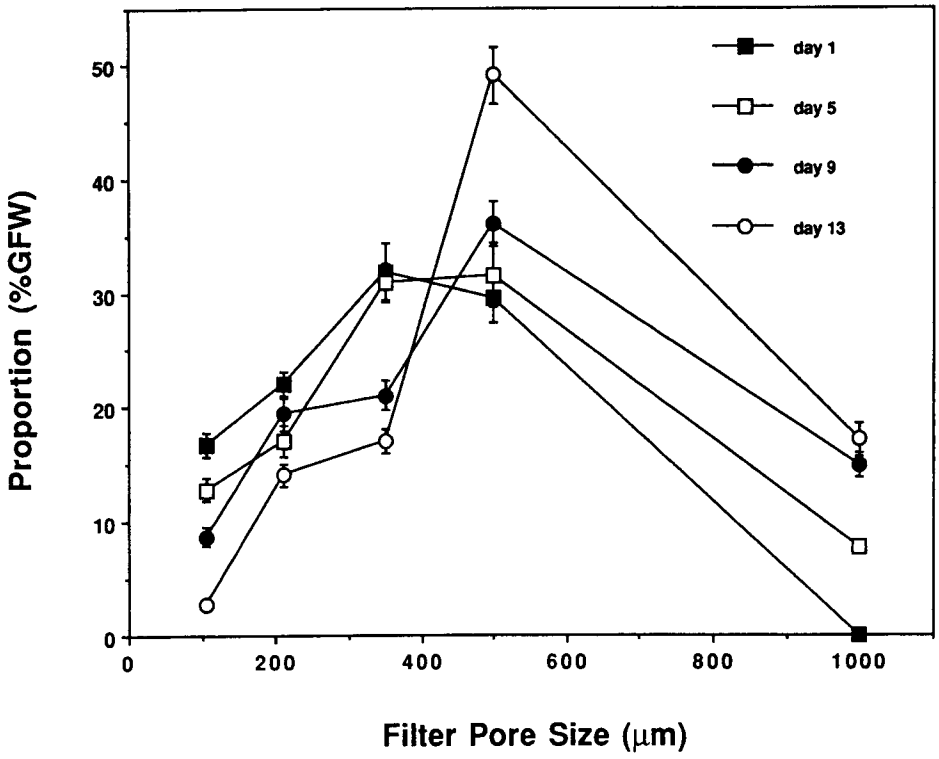

Fig. 6. Changes in cell aggregate size as the culture ages.

line and may provide an important role in limiting clump size. In our hybrid-photobioreactor, mixing is balanced between aeration and impeller speed when the impeller speed is 100 rpm or less. High aeration rates result in deposition of cells as a ring of cells on the glass just above the fluid level. If not washed down daily, a significant reduction in biomass productivity was observed.

To optimize uptake of nutrients by cells and mixing of the medium, suspension cultures consisting of single cells would be ideal. Cell aggregate size of bioreactor cultured cells increased over time, as shown in Fig. 6, for a pair of marine impellers at 100 rpm. Cells cultured in the bioreactor begin to form larger clumps (3–6 mm) late in the exponential phase when cells become sticky). Another factor contributing to clump formation was aggregates washing down from the meringue and biofouling around probes and baffles. The tendency for cell aggregates to increase in size during culture was typical of all impeller types. Biofouling was reduced by the removal of the baffles without apparent loss in mixing efficiency. The reduction in cell aggregate size, observed with a pair of flat bladed turbines, owing to shear damage, resulted in increased cell debris generated from stressed and lyzed cells.

The use of STR for culturing plant cells has been limited to mixing speeds of 100 rpm or less owing to the shear sensitive nature of plant sus-

pensions. Plant cells cultured in bioreactors are often broken or damaged by shear forces caused by aeration, agitation, and pumping. Tanaka et al. (*24*) showed that cells cultured in baffled shake flasks become more resistant to shear stresses caused by hydrodynamic or mechanical forces. Scragg et al. (*25*) developed a cell line of *Catharanthus roseus* tolerant to stirrer speeds of 1000 rpm with good growth in the 150–200 rpm range. They concluded that shear tolerance was not a fixed characteristic, but developed as culture growth improved, suggesting that shear tolerant cells can be selected. Later work by Tanaka et al. (*26*) showed that cells cultured under severe hydrodynamic stress conditions had increased hemicellulose and cellulose. This study suggested that the cell wall in cells cultured under high stress conditions might be physically stronger than those cultured under low stress conditions. We have found that over time, cell lines develop increased tolerance to shear in shake flasks and that these cultures appear to be more tolerant of higher impeller speeds in the bioreactor. When the data in Fig. 5 was compared to data previously presented (*6*), we noted that higher impeller speeds were utilized in this study without apparent damage to the cells. We have recently developed a shear tolerance line that can grow 10% faster than previous lines. It has been cultured in triple baffled 250 mL flasks on an orbital shaker at 200 rpm. This compares to a cell line cultured in a nonbaffled 250 mL flask on an orbital shaker at 120 rpm. Data suggests that suspensions destined to bioreactor culture may be improved by subculturing into baffled 250 mL flasks, increasing shaker speed over time and then selecting for cell lines with higher growth rates.

The percent composition of oven-dried material (105°C) show that the Klason lignin content of soybean cells is low (7.98%). Holocellulose was composed of α-cellulose (20.74%) and hemicellulose (23.03%), resulting in 51.75% of the cell mass as Klason lignin and holocellulose. Other components were starch (6.8%), protein (14.5%), and nucleic acids (6.5%). The high protein and DNA-RNA levels are typical of rapidly-growing young cells. Detroy (*27*) reported that the composition (% dry weight) of the agricultural residue soybean straw was 5.5% protein, 41.4% cellulose, 16.9% arabinose, xylose, mannose and galactose, and no lignin value listed.

One hypothesis was that bioreactor cultured cells would produce a more easily converted substrate. As Table 2 shows, sugar yield after enzymatic hydrolysis of pretreated and untreated biomass did not support this hypothesis. Although 44% of the cell is α-cellulose and hemicellulose, untreated material was recalcitrant to enzyme digestion, yielding only 12.5% of the total cell mass as reducing sugars. Pretreatment with 1 and 5% KOH, even on air dried material, resulted in only 15.44 and 20.66% sugar yields, respectively. Note that 29.2 and 42.9% losses were recorded after 1 and 5% KOH, respectively. Water soluble extractives amounted to 34% of dry wt. Speculation as to the reason for the recalcitrant nature of this biomass would center around was the structure of the cell wall altered.

Table 2
Sugar Yield After Enzymatic Hydrolysis of Pretreated and Untreated Materials

Sample	Treatment	Loss[a], %	Sugar yield, %
Extracellular polysaccharide	none	nd	8.5
Extracellular polysaccharide	5% KOH	27.5	15.7
Whole Cells	water + sonication	34.6	nd
Whole Cells	none	nd	12.5
Whole Cells	1% KOH	29.2	15.44
Whole Cells	5% KOH	42.9	20.66

[a]Percent loss on an oven dried basis after treatment, but before enzymatic hydrolysis.

A white, fluffy, fibrous extracellular material was observed in shake flasks, becoming prominent in 30-d-old cultures, making up 0.5% of the fresh weight. These ECP were composed mostly of noncellulosic materials, such as hemicellulose and callose fibers, that were resistant to cellulase hydrolysis (Table 2). Takeuchi and Komamine (*28*) have shown that extracellular polysaccharides (ECP) were mainly xyloglucans, arabinogalactan, and polyuronide. Auxin or acid conditions induces release of xyloglucans from cell wall preparations (*29,30*). Swelling of this material with 5% KOH followed by enzymatic hydrolysis yielded only 15.7% sugars, compared to nontreated material (8.5%), suggesting the cellulose composition was very low. The material completely dissolved in 24% KOH, suggesting a hemicellulose composition.

CONCLUSIONS

Exploiting fermenter systems for producing biomass will require improving productivity by manipulating key cellular environments: hormone and nutrient regimes, elicitors, shear conditions, medium pH, levels of oxygen and carbon dioxide, temperature, and light. Genetic variation may be used to enhance productivity by developing cell lines with higher levels of hydrolyzable biomass. Value added products could be extractable proteins and secondary metabolites.

ACKNOWLEDGMENTS

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