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Title: Shear Sensitivity and Oxygen Mass Transfer Studies during Cultivation of Tobacco Cells in a Stirred-Tank Bioreactor of Impeller Speeds of 100 to 325 rpm

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Suspension cultures of *Nicotiana tabacum* var Wis. 38 were cultivated in a 3 L Applikon stirred-tank bioreactor to determine the effect of impeller speed (100-325 rpm) on shear damage to cells and oxygen mass transfer rate.

Measurements for viable cell concentration and oxygen mass transfer over a 25 day cultivation period were taken from inoculation through the stationary growth of phase. Tobacco cells were also cultivated within shake flasks under low shear conditions. From semi-log plots of viable cell concentration vs time data, the specific viable growth rate (μ_v) during exponential growth phase and the specific death rate during stationary phase (k_d) were obtained at each impeller speed.

As the impeller speed increased, the final cell density and cell viability decreased. The value for μ_v decreased from 0.175 to 0.097 day^{-1} and the value of k_d increased from 0.042 to 0.109 day^{-1} between impeller tip speeds of 1414 and 4596 cm/sec (initial k_La^0

4.8 hr^{-1} and 19.2 hr^{-1} at 0.425 vvm). In comparision, the value of k_d was 0.036 day^{-1} for low-shear flask cultivation, which was lower than k_d in the stirred-tank bioreactor at impeller speeds of 100 rpm and greater.

During the early stages of cultivation, the value of k_La approximated the initial k_La^0. However, k_La in the culture decreased as the cell density increased. At 250 rpm, the k_La value at a cell density of 8.9 g/L was 7.5 hr^{-1}, versus 11.7 hr^{-1} after the first hour cultivation, a reduction of 36 %. At 175 rpm, the k_La at a cell density of 11.0 g/L was 7.2 hr^{-1}, versus 8.9 hr^{-1} after the first hour cultivation, a reduction of 19 %. However, this reduction in k_La was not sufficient to cause oxygen starvation of the culture.

Shear Sensitivity and Oxygen Mass Transfer Studies during Cultivation of Tobacco Cells in a Stirred-Tank Bioreactor of Impeller Speeds of 100 to 325 rpm

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TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDIX TABLES

NOMENCLATURE

C_A	dissolved oxygen concentration, mmol O_2/L
C^*_A	dissolved oxygen concentration at saturation, mmol O_2/L
D_I	impeller diameter, m
k_d	specific viable death rate, 1/day
k_La	oxygen mass transfer coefficient in culture, 1/hr
k_La^0	initial k_La without cell prescent, 1/hr
N	impeller rotation speed, rpm
t	cultivation time, hr
Qo	volumetric respiration rate of cell culture, mmol O_2/L-hr
X_c	total cell density, g DCW/L
$X_{c,v}$	viable cell density, g DCW/L
v_I	impeller tip speed, cm/sec

Greek letters

μ_v	specific viable death rate, 1/hr
ϕ_v	number fraction of viable cells in the culture sample

Shear Sensitivity and Oxygen Mass Transfer Studies during Cultivation of Tobacco Cells in a Stirred-Tank Bioreactor of Impeller Speeds of 100 to 325 rpm

INTRODUCTION

Plant cells in liquid suspension culture can produce a great variety of specialty chemicals such as pharmaceuticals, flavors, food additives, and fragrances. Currently, most of these chemicals are produced by cultivation of the whole plant, followed by extraction and purification of the desired chemicals. This conventional process has a number of disadvantages: plants are often not available because of geographical or governmental restrictions, and the product yield and quality may be affected by unforeseen enviromental conditions (Panda et al. 1989).

The cultivation of plant cell cultures in bioreactors that provide the best conditions for growth and production of the desired secondary metabolite is an area of active research (Panda et al. 1989). There are only a few commericial scale production process of plant metabolites by plant cells because of technology barriers and high processing cost of industrial scale applications.

Often, significant levels of secondary metabolites are expressed at high cell density during the stationary phase of growth. Unfortunately, the large-scale cultivation of plant cells to high cell density is limited by the sensitivity of indiviual cells, in culture, to hydrodynamic shear stress (Hooker et al. 1989).

For commercial scale production, high cell density is a prerequisite to high volumetric productivity. However, the high cell density can adversely affect the mass transfer rate in the bioreactor system because of insufficient mixing and gas dispersion. Adequate mixing at high cell densities can be achieved by increasing the impeller rotation rate and aeration rate in the pneumatically agitated bioreactors. On the other hand, high levels of aeration and mechanical agitation can cause shear damage of cells, which in turn result in the diminished cell yield and productivity.

Plant cells are shear sensitive in the stationary growth phase because suspension-cultured plant cells have a cellulose-based primary cell wall. During the stationary growth phase, primary cell wall elastically decreases. Furthermore, plant cells increase in size due to accumulation and storage of primary and secondary metabolites. Due to this expansion, stationary-phase cells may be much more shear sensitive than cells in the exponential growth phase (Ballica et al. 1992).

In summary, the large scale cultivation of plant cells to high cell density is not easy: nonuniform mixing, shear damage of fragile plant cells, and limited understanding of oxygen mass transfer are all potential barriers to bioreactor development. In order to address these problems, the specific objectives for this project are to:

1) Investigate cell damage to tobacco cells cultured in a stirred-tank bioreactor during exponential and

stationary phase of growth as a function of impeller speed;

2) Correlate the viable growth and death rates to initial k_La as a fundamental measure of agitation intensity; and

3) Measure oxygen mass transfer (expressed as the culture k_La) at high density.

LITERATURE REVIEW

The following literature review looks at the current state of the art with respect to plant cell culture in bioreactor systems. The review focuses on growth kinetics of plant cell cultures, k_La measurement in cell culture, and shear sensitivity of plant cell cultures.

Growth Kinetics of Plant Cell Cultures

To obtain optimum cell growth and maximum production of secondary metabolites in bioreactor plant cell cultures, several factors, such as bioreactor configuration, impeller design, agitation speed, and aeration rate, must be considered.

Tanka [1981] cultivated *Cudrania tricuspidata* cells in four different type of bioreactors: stirrer tank, modified stirred tank, bubble column, and air-lift reactor. He considered the effect of oxygen supply, intensity of mixing, and air-bubble dispersion on culture growth. Tanaka suggested the most suitable bioreactor for culturing plant cells at high cell density was a jar fermentor with a modified paddle-type impeller, which can yield a cell density of 30 g DCW/L.

Different impeller designs and agitaion speeds affect cultivation of plant cells in a stirred-tank bioreactor. Hooker et al. [1990] cultivated *Nicotiana tabacum* cells in a 5 L stirred-tank fermenter. Hooker et al. found an optimum mixing speed of 150 rpm from a flat blade impeller that produced a maximum wet cell

density of 520 FW g/L. A sail impeller yielded higher growth rates in the exponential phase but increased the lag phase relative to a flat blade impellers.

Aeration rate also affects cultivation of plant cells in stirred-tank bioreactors. Ducos et al. [1986] cultivated *Catharanthus roseus* cells in a 10 L stirred-tank fermenter under aeration rates of 0.4, 1.1, and 1.5 vvm air. The shaft of the fermenter was fitted with a dual pitched-blade turbine impeller. The agitation speed was 80 rpm. They reported that increasing the aeration rate resulted in an extended cultivation time and a lowered final cell density. Gao et al. [1992] cultivated genetically modified tobacco cells in a 5-L stirred tank fermenter (STF) of different aeration rates (0.1, 0.2, 0.7, and 1.1 vvm). They found that cultivation at 1.1 vvm had the highest specific growth rate and reached stationary growth stage more quickly than cultures grown under lower aeration rates.

Other process parameters such as temperature affect plant cell cultivation kinetics. Sahai et al. [1984] investigated the influence of temperature on cultivation of tobacco cells (*Nicotiana tabacum*) in 500-mL Erlenmeyer flasks using 200 mL culture in each flask. They reported that the biomass doubling time at 15 ^{o}C was 210 h compared to 61 h at 27 ^{o}C. At higher temperatures, there was a higher metabolic activity of tobacco cells as evidenced by an increase in total phenolics accumulation.

Sugar level in the medium is also a factor that affects plant cell biomass yields and growth rates. Drapeau et al. [1986] cultivated *D. deltoidea* and *C. roseus* cells in a 14-L fermentor. They reported that the specific growth rates declined steadily during the

latter half of the growth phase, due to the decline in the sugar concentration. Tal et al. [1982] reported that enhanced growth of *D. deltoidea* cell cultures was obtained by raising the initial sucrose concentration from 15 g/L to 60 g/L.

k_La Measurement

The volumetric mass transfer coefficient, k_La, is an index of culture broth mixing and air bubble dispersion in bioreactor systems. Especially, culture k_La measurements are often used to investigate the oxygen transfer rate at high cell density. This culture k_La, and the initial k_La can also be correlated to cell growth rate and maximum cell density. In fact, Bandyopadyay et al. [1967] developed a dynamic method to measure the culture k_La in yeast fermentation systems. This method is based on following the dissolved O_2 concentration during a brief interruption of the aeration.

The values of initial k_La are different under various combinations of agitation and aeration. Kato et al. [1975] used a 15-L jar fermenter with two-flat blade impellers and an air sparger with 17 holes. The initial k_La values for the 15-L fermenter at various aeration rates (0.25 to 1.0 vvm) and agitation speeds (0 to 200 rpm) were determined. The initial k_La was a strong function for both mixing speed and aeration rate.

The initial k_La value can affect the growth of plant cells in batch culture. Gao et al. [1992] investigated the effect of oxygen supply on biomass growth rates in different fermenter

environments. They reported that the initial k_La increased almost linearly with aeration rate in a stirred-tank fermenter at a fixed agitation speed of 150 rpm. Increasing the aeration rate from 0.1 to 1.1 vvm increased the growth rate of tobacco cells. Tanaka [1981] used the initial mass transfer coefficient (k_La) as a measure of the agitation intensity of plant cell suspension cultures grown in shake flasks under various conditions. Tanaka found the growth rate of cells and the yield of cell mass for the 15-day-old culture of *Cudrania tricuspidata* decreased with increasing shaker speed. The yield of cell mass obtained from a 15-day-old culture was also decreased with increasing initial k_La. According to Tanaka's report, increasing initial k_La improved oxygen mass transfer at high cell density but did not increase biomass cell growth rate. Perhaps strong hydrodynamic forces at high agitation speeds resulted in cell damage and prevented continued cell growth.

The presence of cell biomass affects the culture k_La. Tanaka [1981] explained that because the viscosity of culture broth increases steadily due to plant cell growth in the suspension culture, mixing of the broth becomes insufficient, and so air bubbles cannot be dispersed satisfactorily into the culture broth at the high cell density resulting in a reduction in k_La. Agar beads were added in the liquid medium to simulate the presence of cells. Tanaka reported that k_La value at agar bead concentrations of more than 2.0% was reduced considerably relative to the initial k_La in both aeration-type and aeration-agitation-type bioreactor. Ballica et al. [1993] added *Datura stramonium* cells in a 1.2-L draft-tube-type

airlift bioreactor and measured k_La values over an aeration rate range from 0.1 to 1.0 vvm. They developed an empirical correlation

$$k_La = A(U_{gr})^{0.3}(\eta_{eff})^{-0.4}$$

where η_{eff} is the effective viscosity, U_{gr} is the superficial air velocity, and A is the parameter related to the power input. They reported that k_La decreased with increasing cell concentration. The decrease in k_La was attributed to the increasing apparent viscosity of the culture with increasing cell density, and insufficient mixing.

Shear Sensitivity Measurement

Plant cells in liquid suspension culture are considered to be sensitive to hydrodynamic stresses generated by agitation and aeration. This is because plant cells have a relatively large size relative to microorganisms, and possess rigid cell walls and large vacuoles.

Several methods can be used to determine the viability of plant cells under shear treatment, but all have their limitations. Meijer et al. [1993] surveyed methods for determining the effects of hydrodynamic stress on cultured plant cells. They claimed that viability is best defined as the potential of cells to grow and divide when placed in low shear conditions. A major problem with this cell viability assessment is that the time necessary to discern whether or not a cell actually has continued to divide prevents a rapid viability test. Rapid methods commonly used to test viability require that the cell possess an intact ion-impermeable cytoplasmic membrane. Methods for estimating viability by testing membrane integrity are

mostly staining techniques. The major drawback of these methods is their inherent indirectness.

Scragg et al. [1988] used a fluorescein diacetate (FDA) stain method to determine the viability of cell suspensions of *Catharanthus roseus* and *Helianthus annuus* in a 3 L stirred-tank bioreactor. The percentage viability of the cell suspension was determined by counting the number of cells with fluorescent cytoplasm. They found no loss in viability in *Catharanthus roseus* cells after 5 hours shear treatment at a stirrer speed of 1000 rev min^{-1}. Similar results were obtained for *Helianthus annuus* cells at 480 rev min^{-1}. Hooker et al. [1989] used a method developed by Towill and Mazur [1975], which is based on the plant cells' ability to reduce various tetrazolium salts. Reduction occurs when the tetrazolium salt accepts electrons from an electron transport chain in the cell mitochondria. The reduced salt product is a red formazan dye. By measuring the change in absorbance at 485 nm of the red dye present in a given sample, the percent viability was determined. Ballica et al. [1992] used the Evan's blue staining method, where *Datura stramonium* cells were treated with 0.5% (w/v) solution of Evan's blue. The nonviable cells took up the stain, whereas intact and viable cells remained unstained. After staining, viable and nonviable cells were counted on a hemacytometer. The results showed that the viable cell fraction in the concentrated suspension cell culture decreased with increasing applied shear stress during 3 h of shearing.

Shear damage of a plant cell suspension in a hydrodynamic environment can be correlated the specific cell growth rate.

Previous studies have measured the specific cell growth rate during the exponential phase of growth but few have measured the specific cell death rate during the late exponential or stationary phases of growth. Schnapp et al. [1991] measured the specific cell growth rates in batch and semicontinuous culture of *Nicotiana tabacum*. Batch cultures were inoculated from batch stock cultures in the late logarithmic phase of growth. They found that the period of declining specific growth rate or declining μ is extremely brief because of the rapid transition from exponential phase of growth to stationary phase, possibly due to carbon limitation.

Several methods also have been developed to measure the intensity of hydrodynamic stress on cells in culture systems. Hooker et al. [1989] added *Nicotiana tabacum* suspension cells to a Couette-type shearing device to determine the effect of shear rate on culture viability, cell lysis, and the accumulation of secondary metabolites (phenolics). They found that cultures in the latter stages of exponential growth and the early stationary phase of growth were more susceptible to shear damage than cultures in the lag phase, early exponential phase, or later stationary phase. This may be due to cell expansion and growth, along with a decrease in cell wall strength. They also concluded that increasing shear rate caused a steady decrease in viability.

Yang et al. [1992] investigated the inactivation of fragile microalgal cells in the presence of sparging and mechanical agitation. An MTT staining method was used to determine the concentration of viable cells under microscopic examination. A 14-L fermentor was used as the primary working vessel, and a 2-L

fermenter was used as the low shear control vessel. Two six-bladed Rushton turbines, and a single six-bladed Rushtine turbine, were used to stir the 14-L and 2-L fermentors respectively. In the low shear control fermentor, oxygen was provided not by sparging but via surface aeration alone, and agitation was kept as mild as possible in order to minimize cell damage. In the primary fermentor, sparging and agitation were not necessarily maintained at low shear conditions. Both fermentors were simultaneously inoculated with the same initial concentration of the same seed culture, and the specific cell growth rates measurement in both fermentors were also concurrently measured. An equation describing the cellular growth dynamics in an agitated and/or sparged bioreactor was used to analyze viable cell growth from both fermentors. In this equation, the specific growth rate (μ) was eliminated when synchronization of both fermentors was performed. Thus, the specific death rate (p) could be estimated from a plot of $\ln(X(t)/X_c(t))$ versus t for different combinations of operating conditions.

Based on this analysis, Yang et al. [1992] correlated cell death rate (p) with k_La from fragile microalgal cells under various operating conditions. They found that the specific death rate vs k_La data clustered around two linear lines: one under sparging conditions and one under non-sparging conditions. They concluded that increasing k_La increased the specific cell death rate (p).

In summary, biomass density must be optimized to maximize production of secondary metabolites. The growth kinetics of plant cell cultures were well studied by several authors. Increasing agitation speed, aeration rate or both, increases k_La but does not

always increase growth rate. This is because plant cells are shear sensitive under high hydrodynamic force. This is especially true in a stirred-tank bioreactor at high agitation speeds. Some authors focused on the effect of shear stress on plant cell cultures. The plant cells were exposed to a high shear environment for several hours to investigate the percent viability of the cells. No one has investigated the viability of plant cells cultivated in a stirred-tank bioreactor from inoculation through the stationary phase of growth. A major goal of this research is to culture the plant cells until severe cell damage occurs, particulary in the stationary phase. The viable biomass concentration is determined by the product of the total cell biomass density and viability percentage of the culture. The viable biomass vs time data can then be used to determine the specific growth rate of viable cells during exponential phase, and the viable cell death rate during stationary phase. These parameters can be used to access shear damage at different impeller speeds in a stirred-tank bioreactor. Concurrently, the culture k_La is also determined to evaluate the efficiency of oxygen transfer during the whole cultivation process, and to determine if oxygen limitation is potentially a problem at lower agitation intensities.

EXPERIMENTAL METHODS AND DATA CALCULATION

SHAKE FLASK CULTURE

Culture Maintenance

Suspension cultures of *Nicotania tabacum* var Wis. 38 were maintained in 500 mL and 125 mL Erlenmeyer flasks within a Lab-line orbital environmental shaker at 27 °C. Each flask was subcultured approximately every ten days with a 22% to 30% (v/v) inoculum. The conditions for maintaining the culture are found in Table 1. Specific inoculum volumes for each different flask size can be found in Table 2. The MS medium solution was prepared and autoclaved at 121 °C and 15 psig for 20 minutes. The composition of the MS medium is found in Table 3. When subculturing was performed, 40 mL of ten day cells were poured into a new 500 mL autoclaved flask containing 100 mL fresh medium. The mouths of both flasks were flamed to facilitate sterile transfer. The newly subcultured flasks were flamed again and sealed with a foam plug. All subculturing steps were performed in a laminar flow sterile hood.

Table 1. Shake flask culture conditions.

Flask Size (mL) Volume	Shake Speed (RPM)	Total Liquid (mL)
125	150	45
500	120	40

Table 2. Inoculation volumes.

Flask Size (mL)	Medium (mL)	Inoculum (mL)	(v/v)
125	35	10	22
500	100	40	29

Table 3. Culture medium composition in shake flask.

Compound	Amount
MS Medium (Murashige and Skoog Basal) SIGMA Plant Culture Catalog NO. M-5519	4.4 g/L
2,4 - D (2,4-Dichlorophenoxyacetic Acid)	0.1 mg/L
Kinetin (6-Furfurylaminopurine)	0.01 mg/L
Sucrose	30 g/L

BIOREACTOR CULTURE

Bioreactor Description

An Applikon 3 L autoclavable bioreactor with a recommended working volume of 1700 mL was used for the tobacco cell cultivation studies. Figure 1 shows a schematic of the bioreactor.

The 3 L jacketed vessel is made of borosilicate glass. The temperature is maintained by the glass jacket. A water tank with a heating circulator pumps the water through the jacket in order to provide even temperature control. The headplate is made of 316 stainless steel. It has three 6 mm ports, six 10 mm ports, five M 18x 1.5 ports, one G3/4" port, and one central M 30 x 1.0 port. Selected ports on the headplate are fitted with a VirTis galvanic dissolved (D.O.) oxygen electrode, a thermocouple well, an air inlet pipe, and two air outlet pipes. The air inlet and outlet pipes are all equipped with 0.2 μM bacterial air filters. A lipseal stirrer assembly is fitted in the central threaded port of the headplate. This assembly is connected to the stirrer motor. The stirrer motor is controlled by the Applikon model ADI1012 speed controller. The culture is mixed with a three-blade Marine impeller of 45 mm diameter and is connected to an 8 mm shaft. Each blade is pitched to a 45° angle. The air inlet pipe consists of one row of seven 1 mm diameter holes faced toward the bottom of the vessel. Culture samples are withdrawn through a 8 mm sample pipe, and a 1/4 " OD silicon tube. This sample enters a 30 mL autoclaved glass vial. A 60 mL

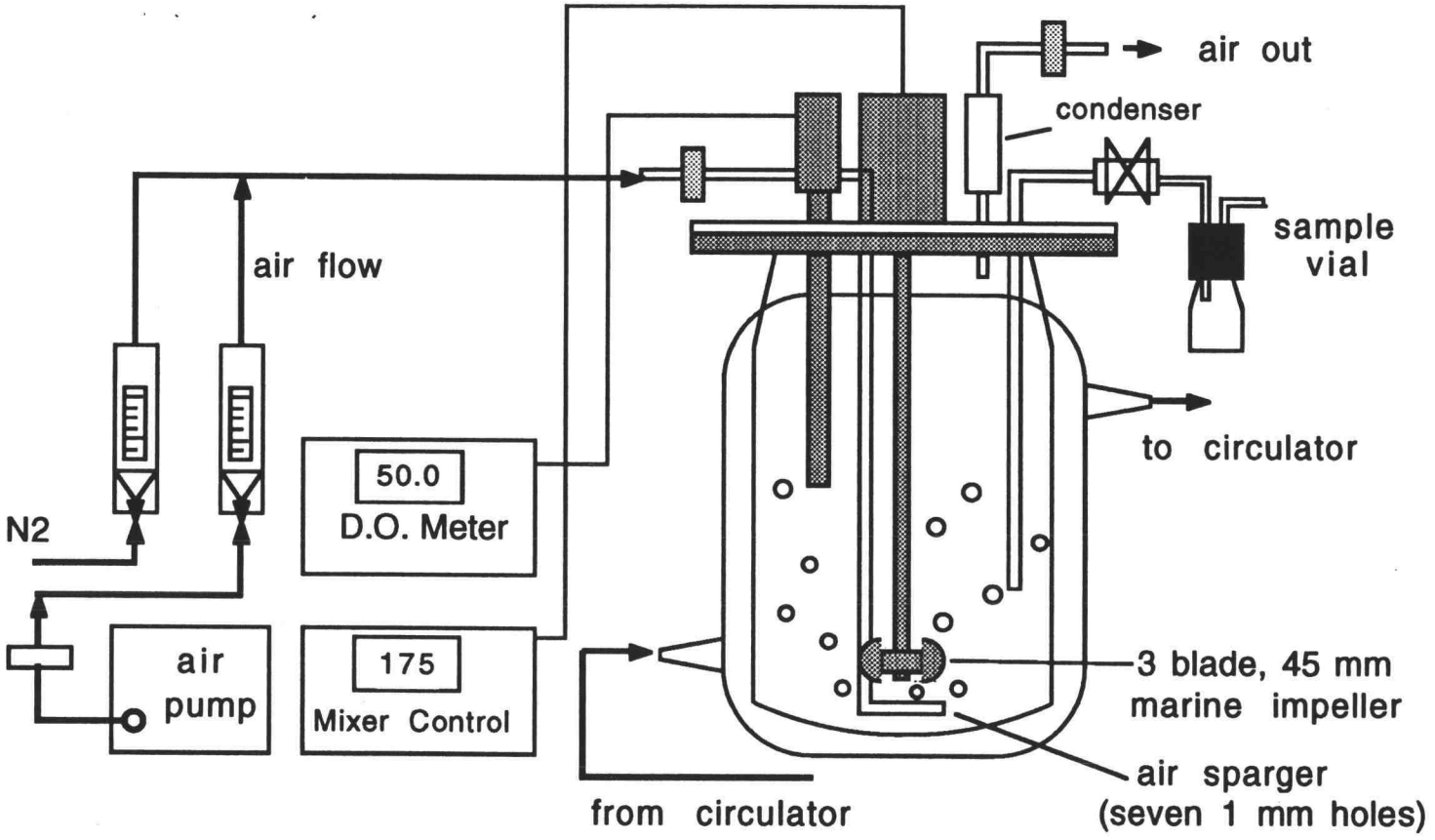

Fig. 1. Three liter Applikon Stirred-Tank Bioreactor

needle syringe is used to provide suction for sample transfer. The silicone tube is clamped except when samples are withdrawn.

The reading from the D.O. meter is sent to an IBM PC/XT computer equipped with a Data Translation DT 2801 I/O Board. Two interfacing programs written in Basic (O2% and DO1) are used to record the D.O. meter output.

Bioreactor Cleaning and Start-Up

The bioreactor was cleaned and sterilized before each new run. The glass and stainless parts were cleaned thoroughly. Hot water and phosphate free detergent were used to clean all parts. After cleaning, the bioreactor headplate and glass vessel were assembled. Then, 1600 mL of distilled water was loaded into the vessel. A clamp was used to close the tubing between the headplate and the air inlet filter. All tubing ends were stuffed with cotton, and covered with foil except for air outlet tubing which was left open during sterilization to maintain pressure equilibrium inside and outside the reactor. The assembled bioreactor (except the motor) was then autocalved at 121° C, 15 psig for 50 minutes.

When the first sterilization was completed, the distilled water was dumped out and 1600 mL MS medium was reloaded into the vessel. Kanamycin (42 mg/L) was added in order to minimize the possibility of bacterial contamination. The assembled bioreactor was then autoclaved again. The MS medium composition is given in Table 4.

Table 4. Suspension culture medium composition in bioreactor.

Compound	Amount
MS Medium (Murashige and Skoog Basal) SIGMA Plant Culture Catalog NO. M-5519	4.4 g/L
2,4 - D (2,4-Dichlorophenoxyacetic Acid)	0.5 mg/L
Kinetin (6-Furfurylaminopurine)	0.03 mg/L
Sucrose	30 g/L
Kanamycin Monosulfate	42 mg/L

After completing the second sterilization, the bioreactor was placed in the laminar flow hood until it cooled down to room temperature. The cooled bioreactor was then connected to the control unit. First, the jacket was connected to the circulation bath, and the air inlet line was hooked up. Second, the lipseal stirrer assembly was connected to the motor. Before inoculation, the D.O. electrode was calibrated at two points. First, humidified air was sparged into the medium for two hours until it was fully saturated with dissolved oxygen. The D.O. electrode was calibrated by setting air-saturated medium reading on the D.O. meter equal to 100%. Second, nitrogen gas was sparged in the medium for one hour in

order to completely strip out the dissolved oxygen. The electrode was then calibrated to 0% on the D.O. meter.

The bioreactor was then disconnected from the control units and placed back in the laminar flow hood. The bioreactor was inoculated in the laminar flow hood with three 500 mL shake flasks, each containing 140 mL of 10 day old tobacco cell suspension. The mouth of each culture flask was flamed, and the cell culture was poured into the bioreactor using a sterilized funnel. The inoculated bioreactor was then connected with the circulation bath, air inlet line, and stirrer motor again. The bioreactor was run under the set conditions. The bioreactor conditions are found in table 5. About one hour was needed to stabilize the temperature and the D.O. level.

Bioreactor Operation

A 25 mL culture sample was withdrawn from the sample pipe every two days from day zero to day six, and then every day from day six to day eight. After day eight, samples were obtained every two days until the run was completed. For each sample, the cell concentration and pH were measured. The number of intact cells, ruptured cells, and cell aggregates was also counted (see Analytical Techniques below).

Table 5. Stirred-tank bioreactor conditions.

Conditions	Settings
Impeller Speed	100, 175, 250, 325 rpm
Air Flow Rate	850 mL/min
Temperature	27 C
MS Medium Volume	1600 mL
Culture Volume	450 mL
Total Volume	2050 mL

Measurements for Qo and k_La were taken once per day. First, the BASIC program O2% was loaded. The current steady state D.O.concentration in the bioreactor was recorded for 5 minutes. The air was then turned off. The D.O. concentration decreased linearly with time due to the oxygen demand of the cell culture. When the D.O. concentration decreased to 20 %, the air was then turn backed on. The D.O. concentration then returned to the current steady state level with time. The above procedure was repeated to get duplicated measurements.

The D.O. concentration was recorded continuously during the entire bioreactor run except when measurements for Qo and k_La were taken. The estimation of Qo and k_La from D.O. concentration vs time data is described at the end of this chapter.

ANALYTICAL TECHNIQUES

Cell Concentration

Cell concentration was measured as the dry cell weight and packed cell volume. Two 10 mL aliquots from the glass sample vial were poured into 15 mL conical centrifuge tubes. The samples were centrifuged at 2000 rpm (523 g) for 15 minutes using a IEC Centra 4B centrifuge. The supernate was withdrawn by a Pasteur pipet from the centrifuge tube and then measured in a 10 mL volumetric cylinder. The packed cell volume (PCV, volume of cells/culture volume) was determined by the difference between the culture volume and supernate volume divided by culture volume. The

centrifuged cell mass was taken out by a spatula and put on a dried, pre-weighed disk of Whatman filter paper #1 (55 mm diameter). The residual cells in the centrifuge tube were resuspended in distillled water and then slowly poured on the same Whatman filter paper. The filter paper and wet cell filter cake were then dried at 70 ^{o} C for 48 hours. The dry cell density (g dry cell/L culture) was determined by the difference between the filter paper with and without cell mass, divided by the original sample volume.

Cell Counting under Microscope

The cell culture was visually inspected for intact and damaged cells using an AO/Spencer bench top microscope under 100X and 430X magnification. About 0.5mL of the culture sample was mixed with Evan's blue stain (0.25 wt%). The stained culture sample was loaded into a Fuchs-Rosenthal hemocytometer (1/16 mm^2 per square and 2/10 mm deep) with a Pastuer pipet. A separate sample was placed on a glass and inspected under the microscope for contamination. The stained culture sample in the hemocytometer was placed under the microscope and counted on 16 triple ruled squares ($1mm^2$) for the number of intact cells, ruptured cells, and cell aggregates. Dead and ruptured cells were converted to blue color. Intact cells were not stained and did not show the characteristic blue color. The number fraction of cells was determined by the number of intact cells, ruptured cells, and cell aggregates divided by the total number.

pH Measurement

The pH of each culture sample before centrifugation was measured by a Corning 109 digital pH meter and a general purpose electrode.

Initial k_La Measurement

Initial k_La measurement were taken after calibrating the D.O. electrode. The liquid volume was 2000 mL of MS medium. First, the desired impeller speed was set and humidified air was sparged into the vessel until 100% saturation of the liquid was attained. The oxygen was stripped out by sparging with nitrogen gas until 50% of air saturation was obtained. The nitrogen gas was turned off, and the remaining N_2 bubbles in the medium were allowed to vent out. The air was then turned on again. The dissolved oxygen concentration was recorded continuously as a function of time by the D.O. electrode.

If no cells are present and the liquid phase is well-mixed, then the dissolved oxygen balance is given by

$$\frac{dC_A}{dt} = k_La[C^*_A - C_A] \qquad [1]$$

where C_A is the dissolved oxygen concentration (mmol O_2/L) and C^*_A is the dissolved oxygen concentration at saturation. Integration of equation 1 from an initial condition $t = t_0$, $C_A = C_{A0}$ gives

$$\ln \left[\frac{C^*_A - C_A}{C^*_A - C_{A0}} \right] = -k_L a^0 [t - t_0] \qquad [2]$$

where $k_L a^0$ is the initial volumetric mass transfer coefficient. Thus, The initial $k_L a^0$ value was determined from the least-squares slope of a semi-log plot of D.O. concentration vs time data during the re-aeration phase of the experiment from equation 2. The effect of impeller speed on initial $k_L a^0$ at an aeration rate of 0.43 vvm and temperature of 27 ^oC for the Applikon 3-L stirred-tank bioreactor is shown in Figure 2.

D.O. Electrode Maintanence

A steam sterlizable VirTis galvanic dissolved oxygen electrode was used to measure the D.O. concentration in the cell culture. After sterilizing and before inoculating, the electrode was refilled with electrolyte. A syringe was used to inject the electrolyte into the filling tube until there are no air bubbles coming out from the vent tube. After the electrode was used for a period of time (4 - 5 autoclavings), impure residue sometimes formed on the cathode and anode. These residues were cleaned to maintain the electrode.

Estimation of Qo and $k_L a$

The respiration rate Qo and volumetric mass transfer coefficient $k_L a$ in the bioreactor culture are estimated by a dynamic measurement technique (Figure 3). The unsteady-state mass balance on dissolved oxygen in cell cultures is

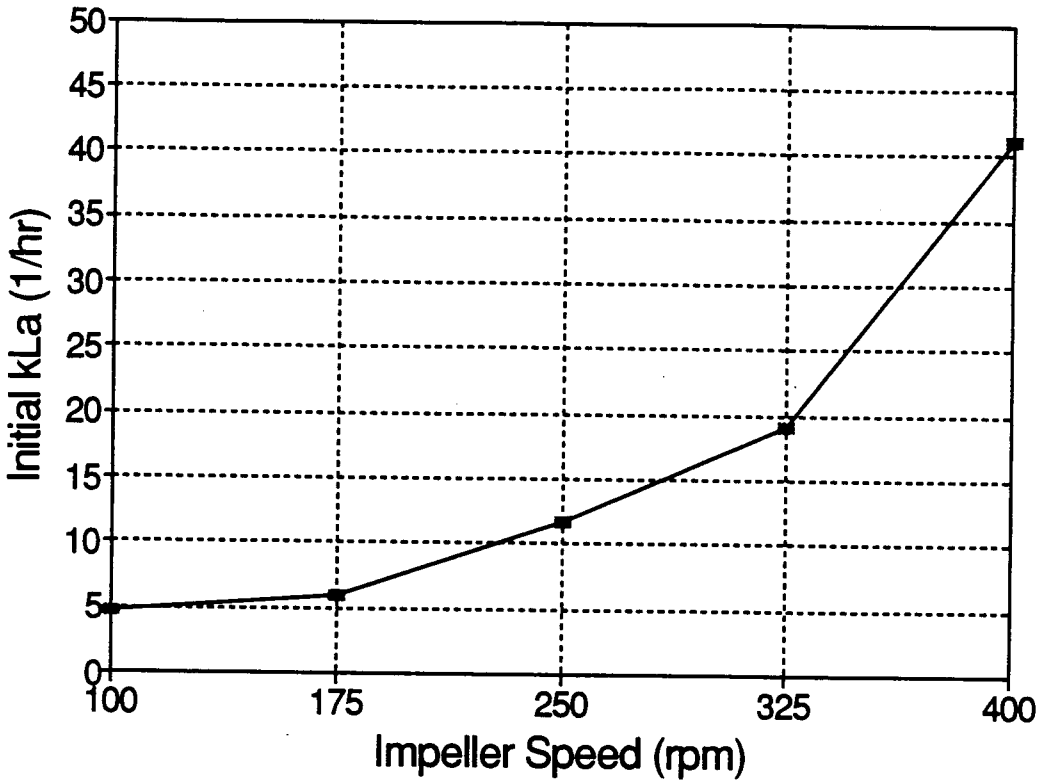

Figure 2. Initial k_La vs. mixing speed at 0.43 vvm aeration rate.

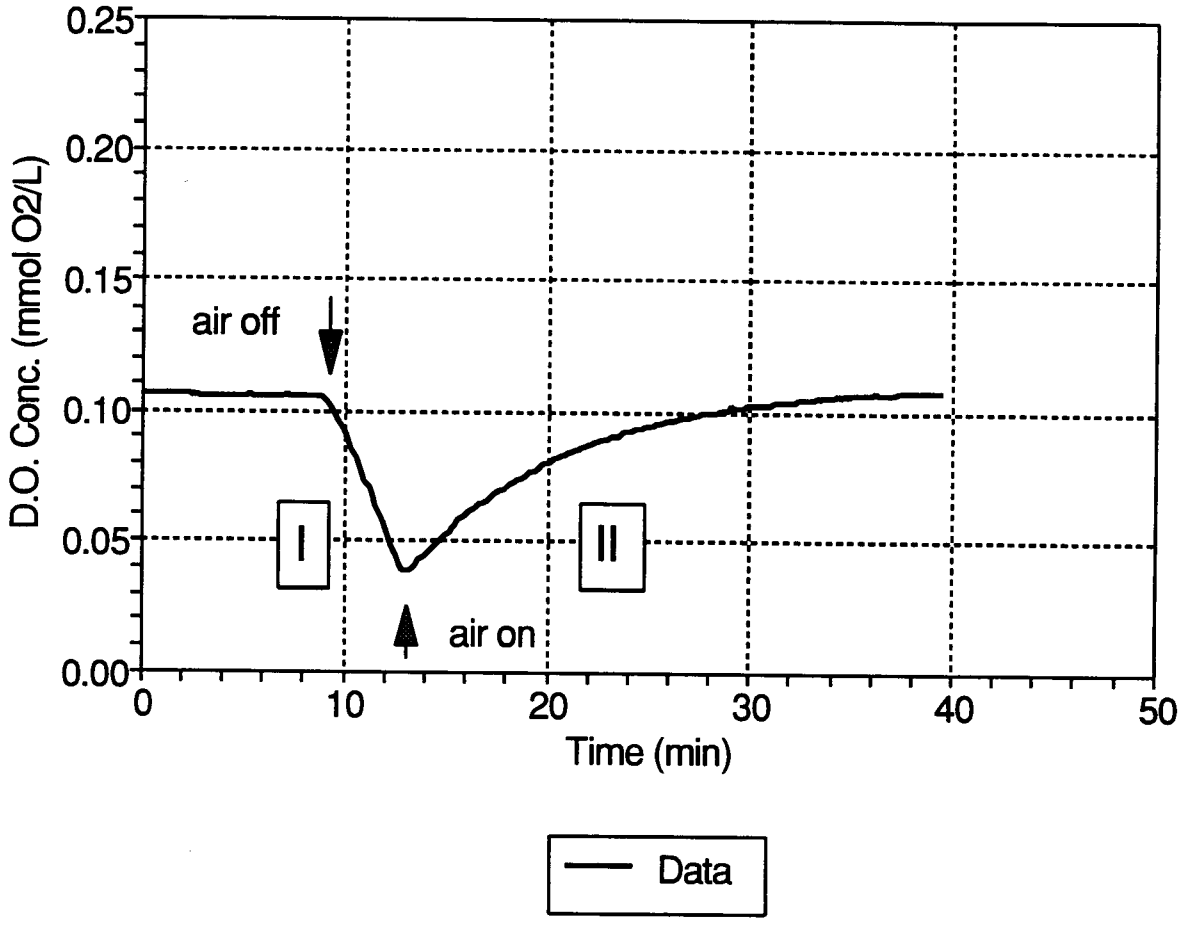

Figure 3a. Estimation of Qo and culture k_La by the dynamic method Cultivation conditions: impeller speed, 175 rpm; aeration rate, 0.43 vvm; cultivation hour, 91 hr; total cell density, 5.4 g DCW/L.

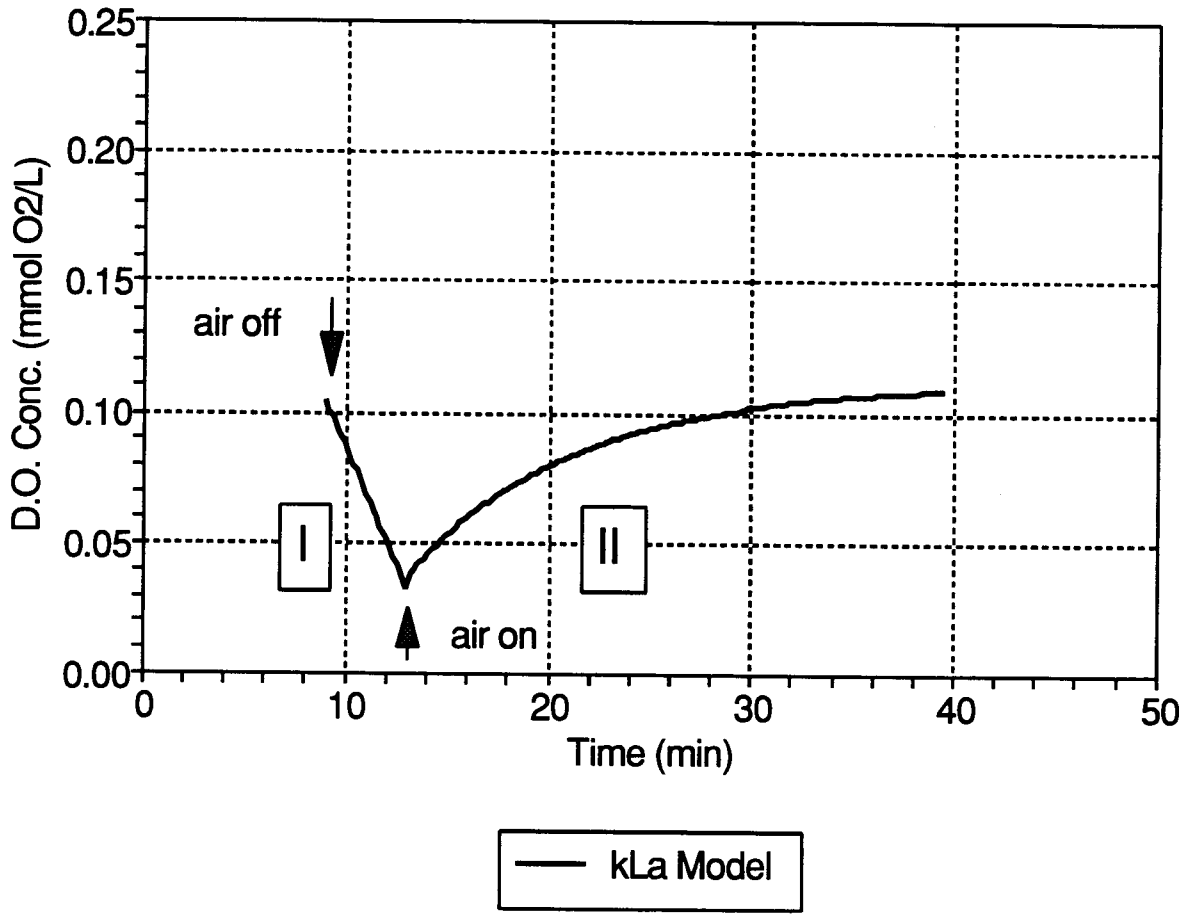

Figure 3b. Estimation of Qo and culture k_La by the dynamic method Cultivation conditions: impeller speed, 175 rpm; aeration rate, 0.43 vvm; cultivation hour, 91 hr; total cell density, 5.4 g DCW/L.

$$\frac{dC_A}{dt} = k_La[C^*_A - C_A] - Qo \qquad [3]$$

When the aeration supply to the bioreactor is turn off (Figure 3, stage I), the dissolved oxygen balance reduces to

$$\frac{dC_A}{dt} = -Qo \qquad [4]$$

and Qo is estimated from the least-squares slope of the D.O. concentration (C_A) vs. time data. When the aeration supply is turned back on (Figure 3, stage II), the dynamic response curve is found by analytical integration of equation 3:

$$C_A = C^*_A - \frac{Qo}{k_La} - [C^*_A - \frac{Qo}{k_La} - C_{A1}]\, e^{-k_La(t-t_1)} \qquad [5]$$

where C_{A1} at time t_1 is the D.O. concentration at the moment the aeration supply is turned back on. If Qo is known from stage I, then k_La is the only unknown parameter in equation 5. The k_La value then can be estimated by minimizing the sum of squared deviations between model (equation 5) and the D.O. concentration vs time data, i.e.

$$S^2 = \sum_{i=1}^{n} (C_A(t_i) - C_{Ai})^2 \qquad [6]$$

where $C_A(t_i)$ is the model prediction for the D.O. concentration by equation 5, and C_{Ai} is the measured D.O. concentration at time t_i.

The optimization algorithim in Quattro-Pro version 4.0 software was used to iteratively perform the least-squares minimization.

RESULTS AND DISCUSSION

The results presented here show the effect of mixing speed on growth and shear sensitivity of *N. tabacum* cell suspension cultures in the Applikon 3 L stirred-tank bioreactor. Specifically, the specific viable cell growth rate (μ_v) in exponential growth phase and the specific death rates (k_d) in stationary phase are correlated to impeller speed and initial k_La. The effect of impeller speed on oxygen mass transfer rate during the entire cultivation period is also presented.

EFFECT OF MIXING SPEED ON CELL CULTURE GROWTH AND SHEAR SENSITIVITY

Growth Curve

Growth curves for tobacco cells cultivated in the Applikon 3-L stirred-tank bioreactor at a fixed aeration rate of 0.43 vvm for impeller speeds of 100, 175, 250, and 325 rpm are presented in Figures 4 and 5. The growth curve for tobacco cells cultivated in 125 mL shake flasks at 150 rpm orbital rotation speed is also presented in Figure 4.

The total biomass density (X_c) was measured as dry cell weight per liter of culture (g DCW/L). The highest cell density of 12.9 g DCW/L was obtained from the flask cultivation after 347 hr (14.5 days). For the stirred-tank bioreactor, a maximum cell density of 11.8 g DCW/L was obtained at 100 rpm impeller speed

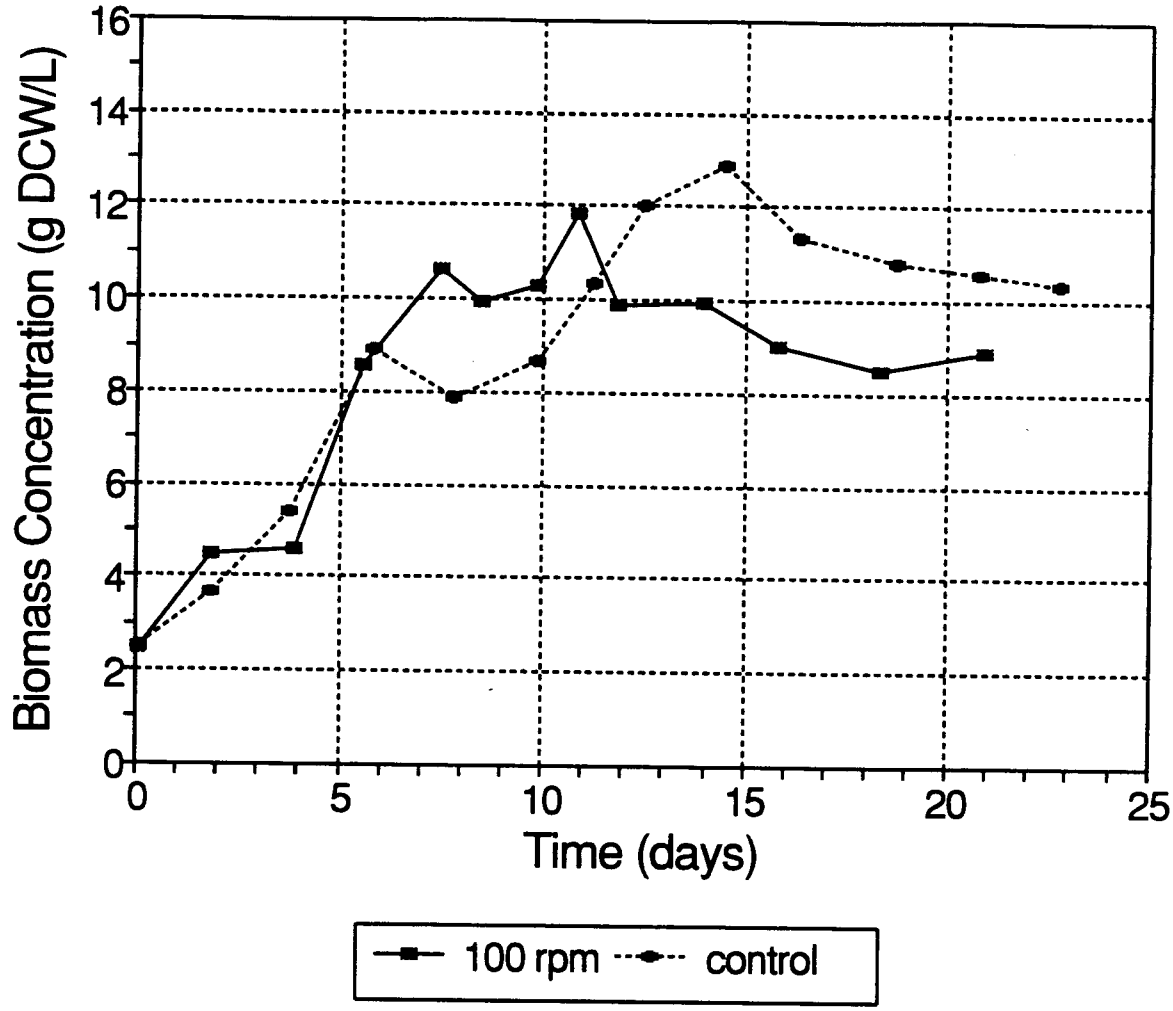

Figure 4. Growth curve at 100 rpm and in shake flasks.

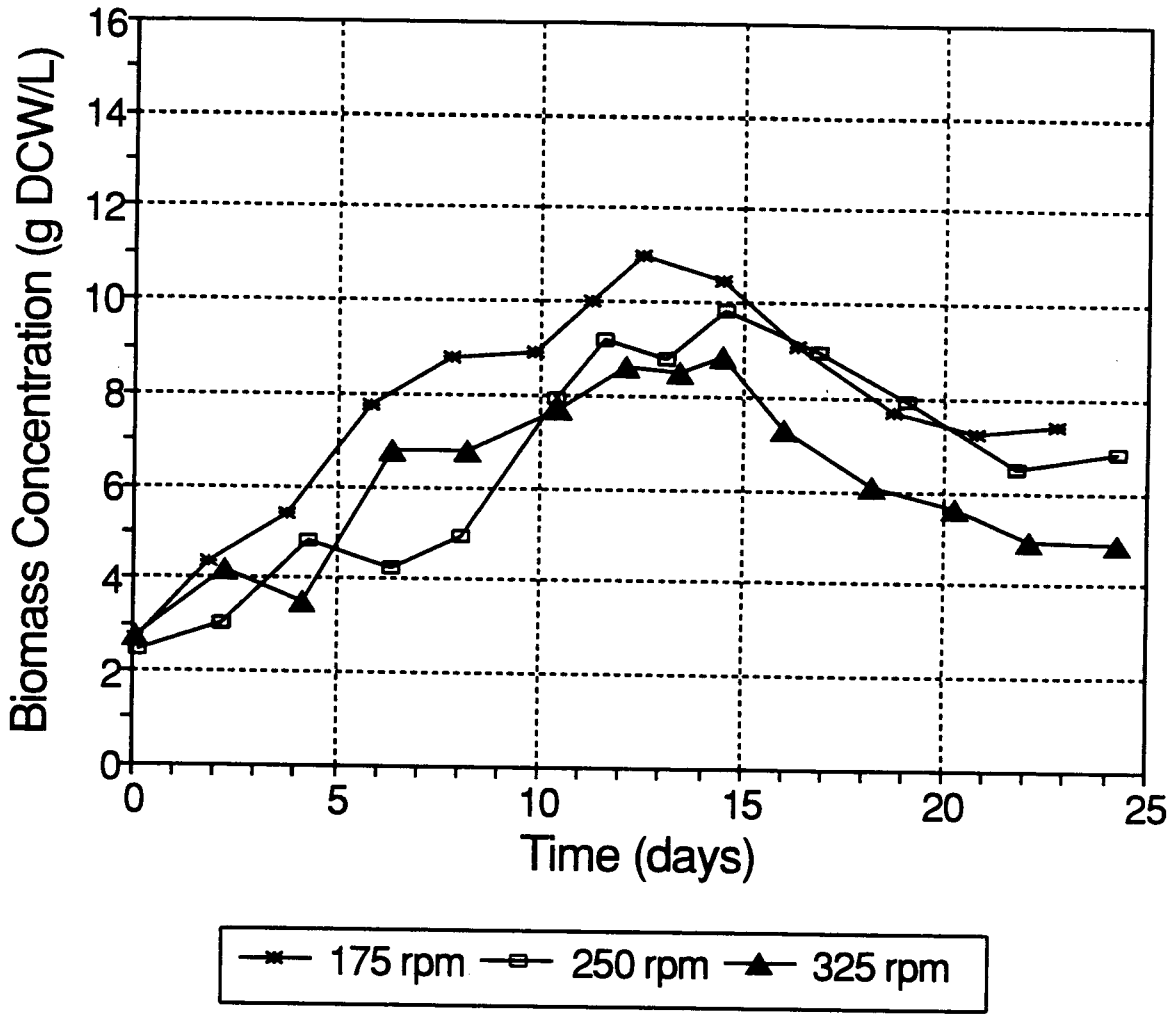

Figure 5. Growth curve at 175, 250, and 325 rpm.

after 261 hr of cultivation (10.9 days). However, at an impeller speed of 325 rpm, the maximum cell density was only 8.9 g DCW/L. The cell density of entrained cell on the wall was 3.25 g DCW/L after completion of the cultivation at an impeller speed of 325 rpm, while the cell density in cell cultures was 4.87 g DCW/L. In general, increasing impeller speed depressed the maximum cell density. This trend suggested that hydrodynamic shear forces at high impeller speeds were damaging the cells in the culture, leading to the loss of biomass productivity and culture viability.

Number Fraction of Viable Cell vs Culture Time

The number fraction of viable cells in the stirred-tank bioreactor was measured as function of cultivation time and impeller speed as shown in Figures 6 and 7. The number fraction vs time profile of viable cells grown in a low-shear environment within 125 mL flasks on an orbital shaker at 150 rpm is also shown in Fig 6. In the bioreactor, the number fraction of viable tobacco cells was 88 % at 100 rpm, versus 66.5 % at 325 rpm after the first hour of bioreactor cultivation. However, the number fraction of viable cells cultivated in 125 mL flasks was 95.5 % after the first hour of cultivation. In fact, the viability of the shake flask culture was always higher than the viability of the bioreactor culture over the entire cultivation period, even at a low mixing speed of 100 rpm.

Each curve of Figures 6 and 7 seems to have three periods of rapid decrease in % cell viability. For example, at 250 rpm, the number fraction of viable cells rapidly decrease during the first four

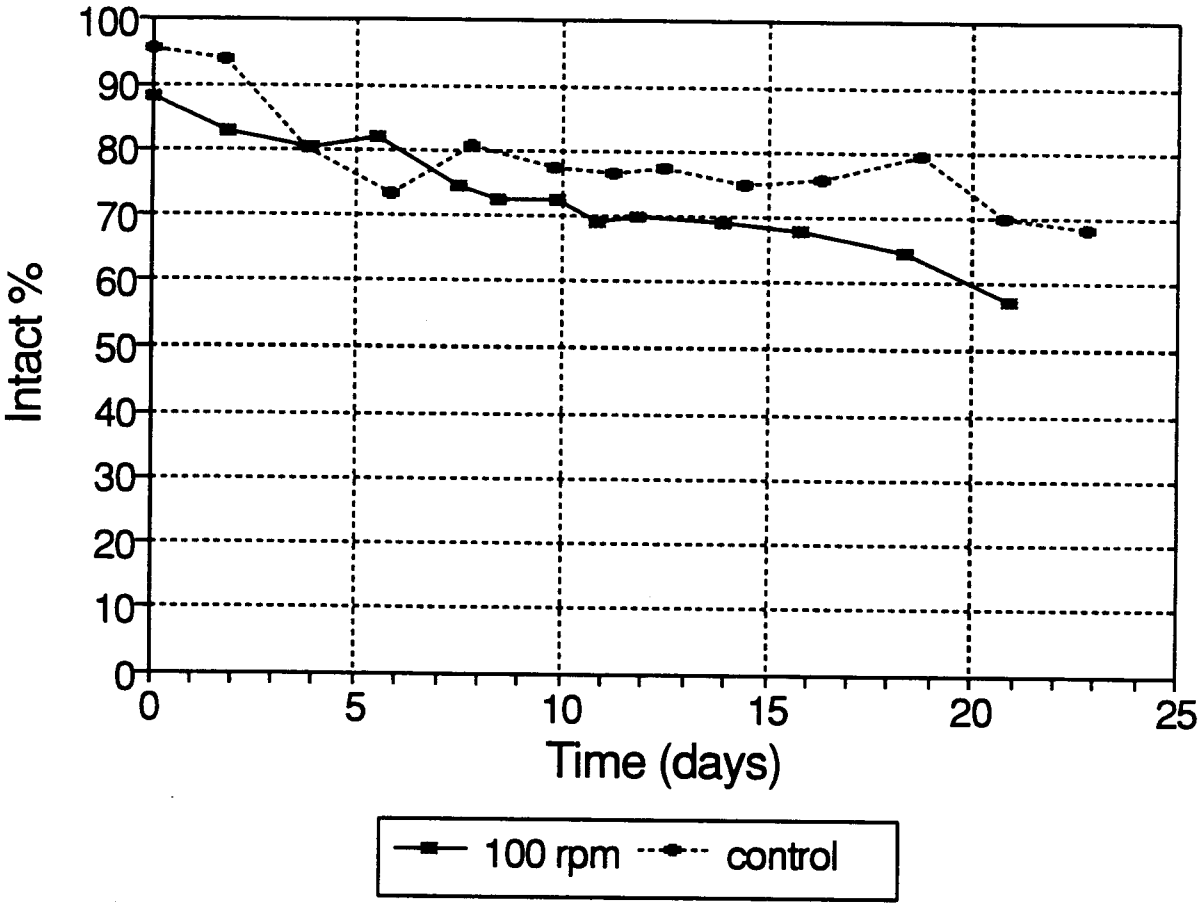

Figure 6. Number fraction of viable cells vs. cultivation time at 100 rpm and in shake flasks.

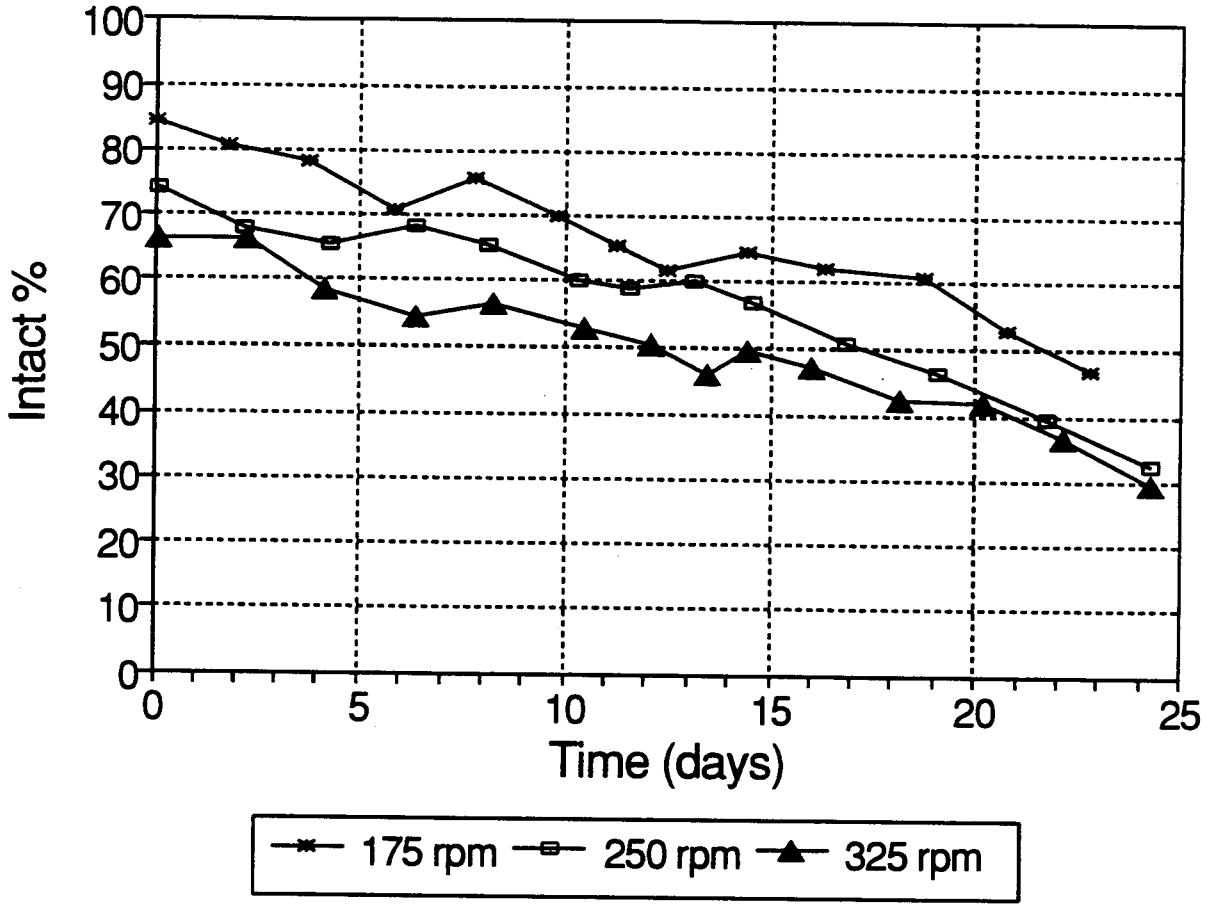

Figure 7. Number fraction of viable cells vs. cultivation time at 175, 250, and 325 rpm.

days of cultivation, decrease rapidly again from day 7 to day 10, and then also rapidly decrease from day 14 to day 24. Also, the % viability vs time curve decreases with increasing impeller speed of 100 to 325 rpm.

Microscopic analysis of tobacco cells in the stirred-tank bioreactor culture revealed an increase in the number of visibly-ruptured cells, cells with deformed morphology, and cell debris relative to flask cultures of comparable age and cell density.

Specific Cell Growth (μ_v) and Death Rate (k_d)

In order to assess hydrodynamic shear damage to tobacco cell suspension cultures grown in a stirred-tank bioreactor, it is appropriate to estimate the viable cell density as a function of cultivation time and process conditions. Previous studies in stirred-tank bioreactors only considered the loss in total biomass density with increasing agitation intensity (Kato et al., 1975; Hooker et al., 1990; Leckie et al., 1991). A simple relationship for estimating viable cell density ($X_{c,v}$) is

$$X_{c,v} = \phi_v X_c \qquad [7]$$

where ϕ_v is the number fraction of viable cells in the culture sample.

The semi-log plots of $X_{c,v}$ and X_c vs. time for tobacco cells cultivated in shake flasks and in the stirred-tank bioreactor at impeller speeds of 100 to 325 rpm are shown in Figures 8-12. In each semi-log plot, the viable biomass concentration linearly

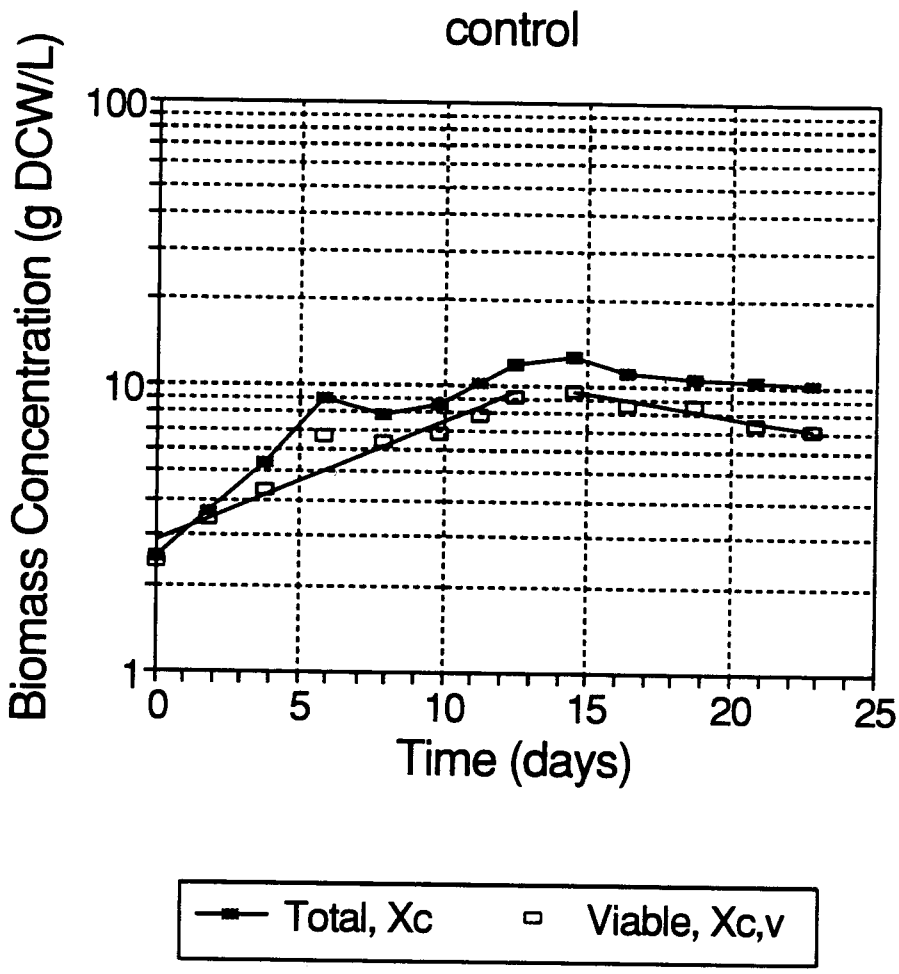

Figure 8. Semi-log plot of X_c and $X_{c,v}$ vs. time in shake flasks.

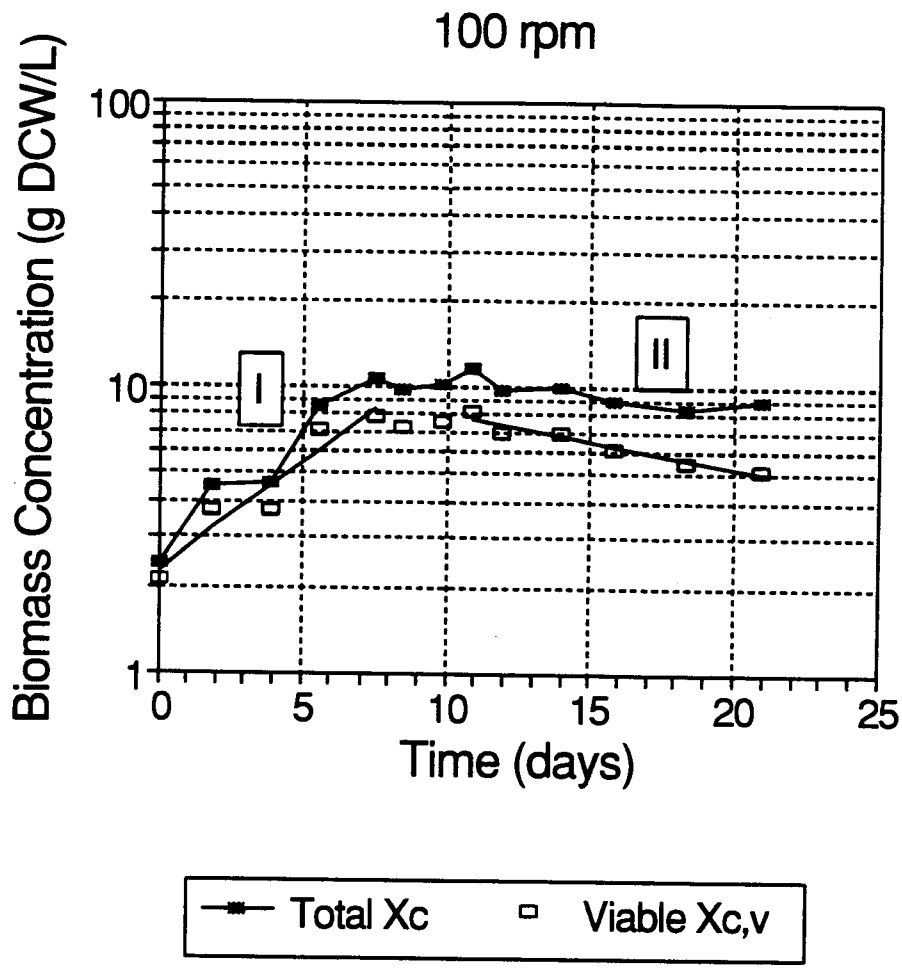

Figure 9. Semi-log plot of X_c and $X_{c,v}$ vs. time at 100 rpm.

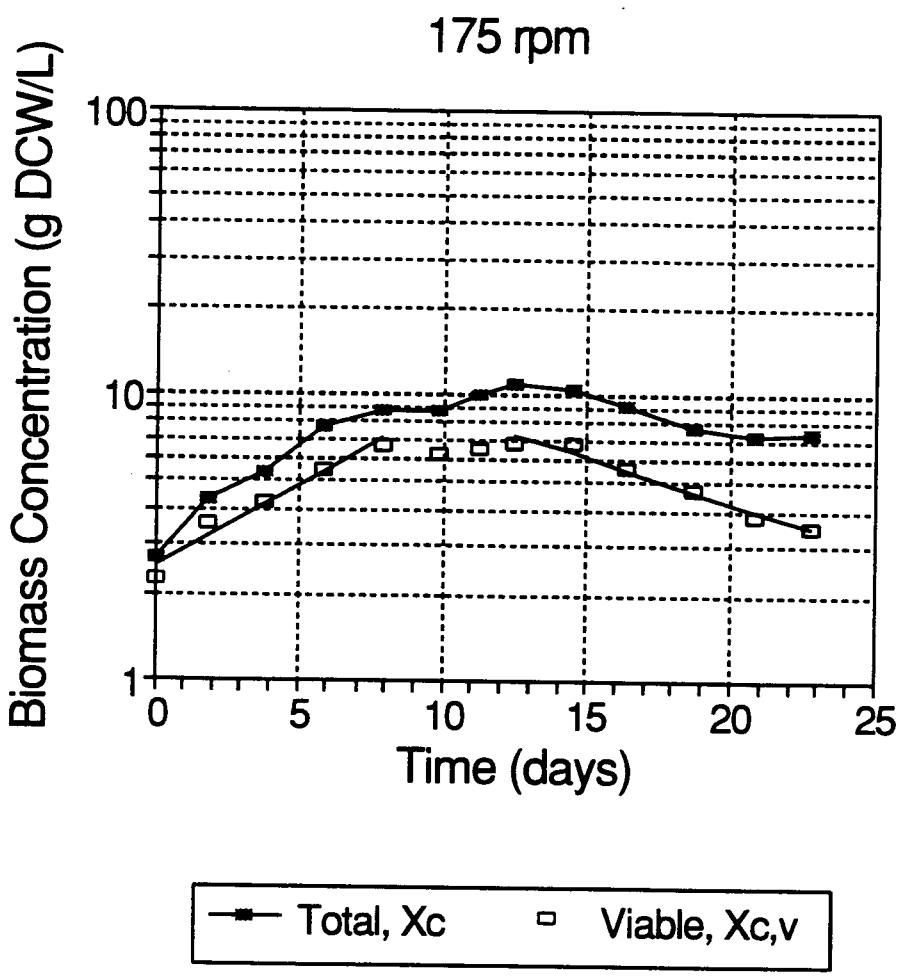

Figure 10. Semi-log plot of X_c and $X_{c,v}$ vs. time at 175 rpm.

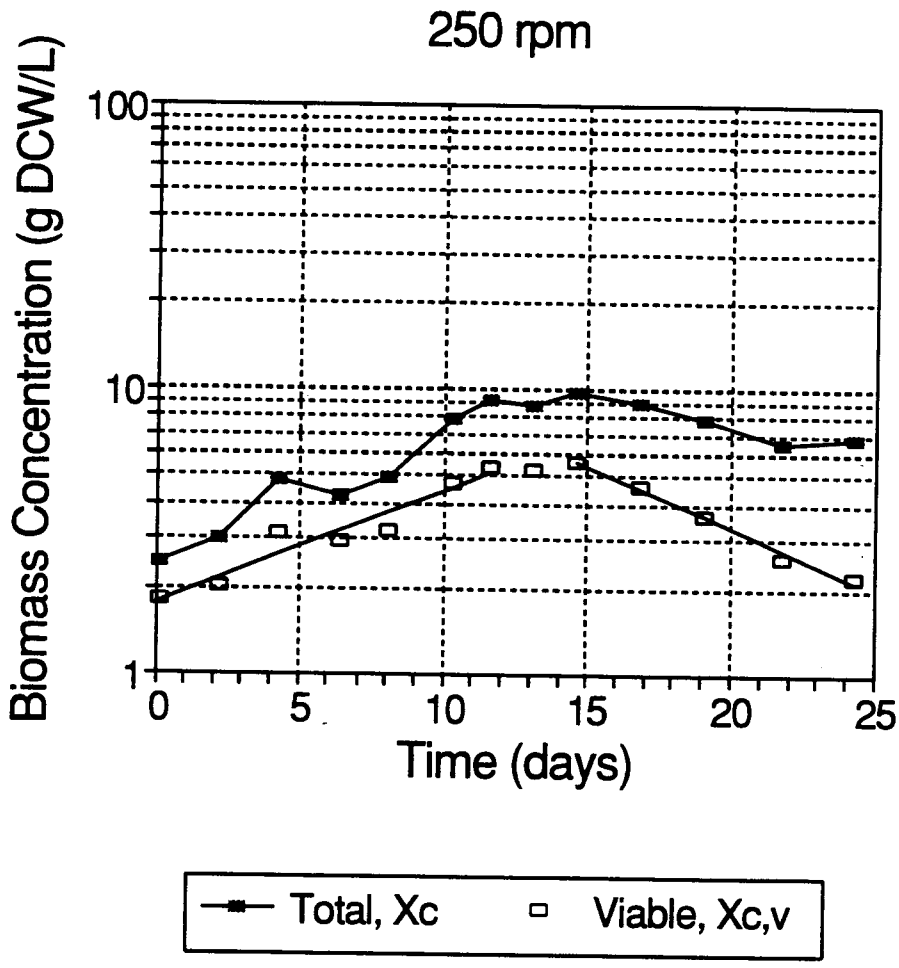

Figure 11. Semi-log plot of X_c and $X_{c,v}$ vs. time at 250 rpm.

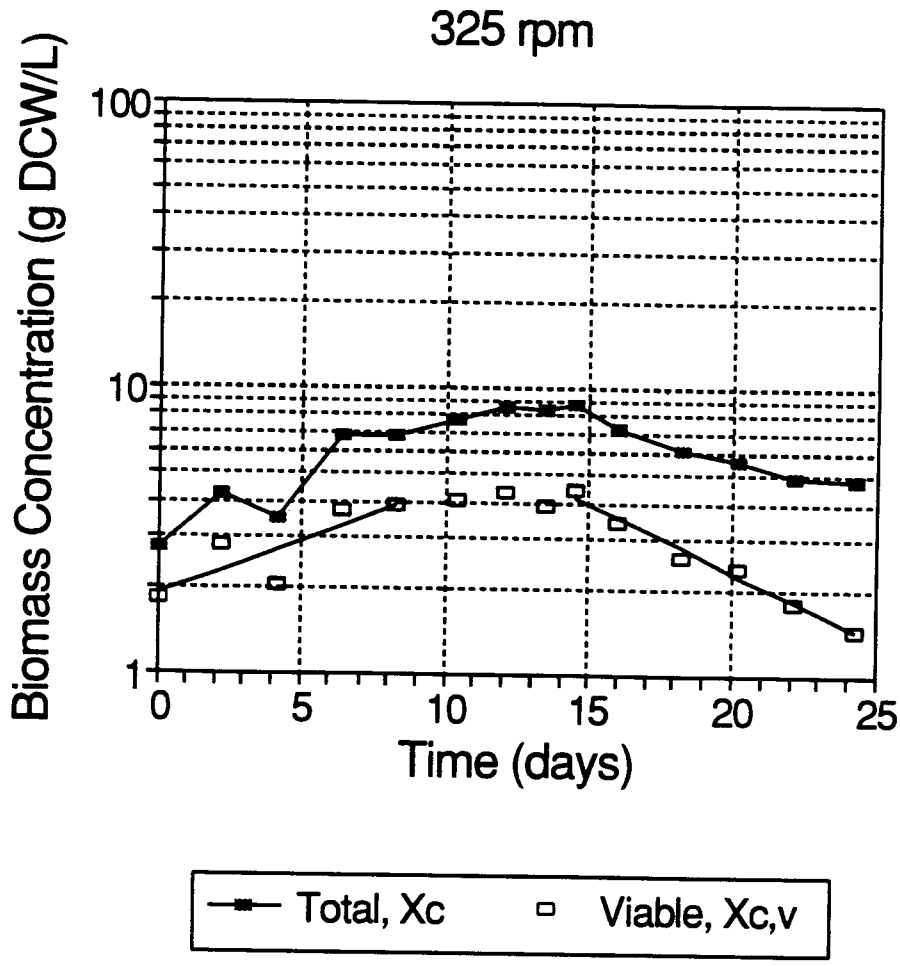

Figure 12. Semi-log plot of X_c and $X_{c,v}$ vs. time at 325 rpm.

increased during the exponential growth phase starting from the time of inoculation, implying that no significant lag phase was observed. When the viable biomass concentration approached the maximum density, the cell culture growth slowed down and several days were needed to reach the maximum viable cell density. After reaching the maximum viable cell density, the cell cultures shifted to the stationary phase of growth, and the viable cell density decreased linearly on the semi-log plot.

The viable cell density leveled off before the total biomass density leveled off at impeller speeds of 100 to 325 rpm. The reason is that the number fraction of intact cells decreased during the whole cultivation periood. Thus, the value of $X_{c,v}$ is always smaller than the value of X_c.

Also, the semi-log plots of $X_{c,v}$ vs. time (Figure 9) reveal two distinct linear regions (I and II). Region I represents the exponential phase of growth and has a positive slope μ_v (day^{-1}), where μ_v is the net growth rate of viable cells in a culture phase where cells are actively dividing. Region II represents the stationary phase of growth and has a negative slope $-k_d$ (day^{-1}), where k_d is the specific death rate of viable cells in a culture phase that are not actively dividing. Points for data regression were selected only where the data was linear on the semi-log plot. Thus, for the exponential phase, the rate of viable culture growth is given by

$$\frac{dX_{cv}}{dt} = \mu_v X_{cv} \qquad [8]$$

and the rate of viable biomass decrease during the stationary phase of growth is given by

$$\frac{dX_{cv}}{dt} = - k_d X_{cv} \qquad [9]$$

At an impeller speed of 325 rpm, the exponential growth period shortened to 198 hr (8.3 days), relative to 278.5 hr (11.6 days) at 250 rpm. At 325 rpm, The stronger hydrodynamic forces shortened the exponential growth period and the maximum viable cell density was only 4.4 g DCW/L.

After the exponential growth phase, an additional 3 to 5 days were still needed to reach the maximum cell density at impeller speeds of 100, 175, and 250 rpm. However, an additional seven days were required at a cultivation speed of 325 rpm. At an impeller speed of 100 rpm, 261 hr (10.9 days) were needed to reach the maximum viable cell density of 8.2 g DCW/L. When the impeller speed was increased from 100 to 250 rpm, longer times were required to reach maximum viable cell density.

The effect of impeller speed on μ_v and k_d are shown in Figures 13 and 14 respectively. In Figure 13, as the impeller speed increased, the specific viable growth rate (μ_v) decreased rapidly between 100 rpm and 250 rpm, and then more slowly from 250 rpm to 325 rpm. At the impeller speed of 325 rpm, the maximum viable cell density was also lower than that of the impeller speed 250 rpm. However, μ_v had a slightly higher value at 325 rpm as that at 250 rpm. As shown in Figure 14, as impeller speed increased, the

specific viable death rate (k_d) increased rapidly between 100 rpm and 250 rpm and then more slowly from 250 rpm to 325 rpm.

The specific cell death rate k_d is a lumped parameter which quantifies loss in viable cell density due to both hydrodynamic shear damage (necrosis) and natural necrobiosis of the cell culture during stationary phase. Therefore, in the low-shear flask cultivation environment, k_d should primarily represent natural necrobiosis of the culture. Thus, the increase in k_d with increasing agitation intensity in the stirred-tank bioreactor can be attributed mainly to hydrodynamic shear damage, because the value for k_d during flask culture experiment was only 0.036 1/day, which was at least a factor of two lower than k_d in the stirred-tank bioreactor at impeller speed greater than 175 rpm.

In summary, *N. tabacum* cells were sensitive to hydrodynamic shear at impeller speeds of 100 to 250 rpm. However, at a higher impeller speed of 325 rpm, cells may have adjusted to hydrodynamic shear and became more resistant to shear.

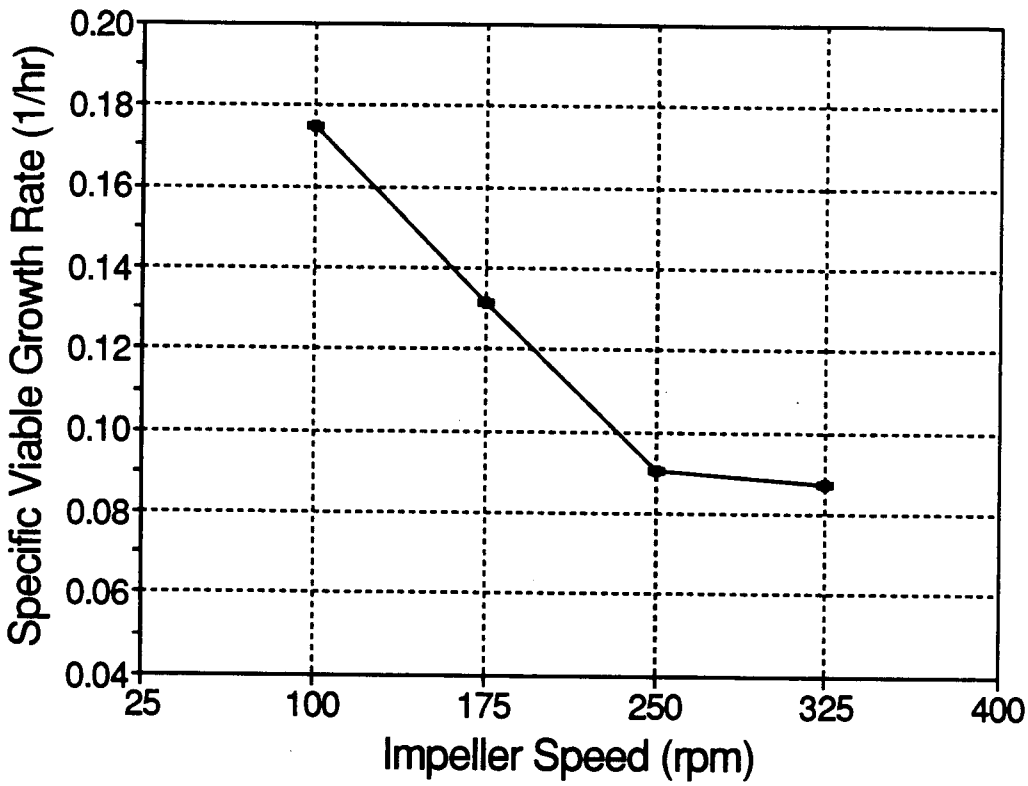

Figure 13. Specific viable growth rate (μ_v) vs. impeller speeds of 100 to 325 rpm.

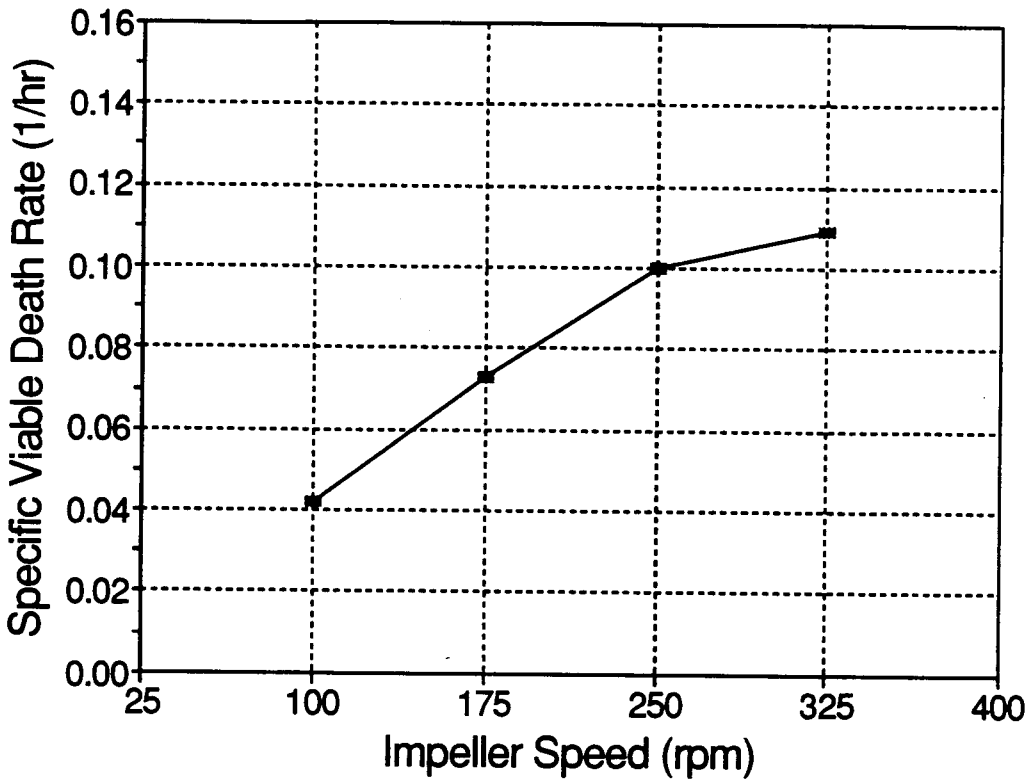

Figure 14. Specific viable death rate (k_d) vs. impeller speeds of 100 to 325 rpm.

Correlation of μ_v and k_d to $k_L a$

Figures 13 and 14 show that the tobacco cell suspension culture is suspectable to hydrodynamic shear damage in the stirred-tank bioreactor during all phases of growth. Furthermore, μ_v decreases and k_d increases with increasing agitation intensity. Ideally, μ_v and k_d should be correlated to hydrodynamic shear stress. However, the hydrodynamic shear stresses in a stirred-tank bioreactor are difficult to quantify during batch cultivation of plant cells to high cell density. This is because 1) the hydrodynamics in a stirred-tank bioreactor are inherently complex and hard evaluate, and 2) the cells are subjected to continuously changing hydrodynamic stress conditions imposed by changing rheology of the culture as cell biomass concentration increases (Meijer et al., 1992).

Instead, μ_v and k_d were correlated to v_I, the impeller tip speed for the stirred-tank bioreactor, as shown in Figures 15 and 16. Values for v_I were calculated by

$$v_I = \pi N D_I \qquad [10]$$

where D_I is the impeller diameter, N is the impeller rotation speed.

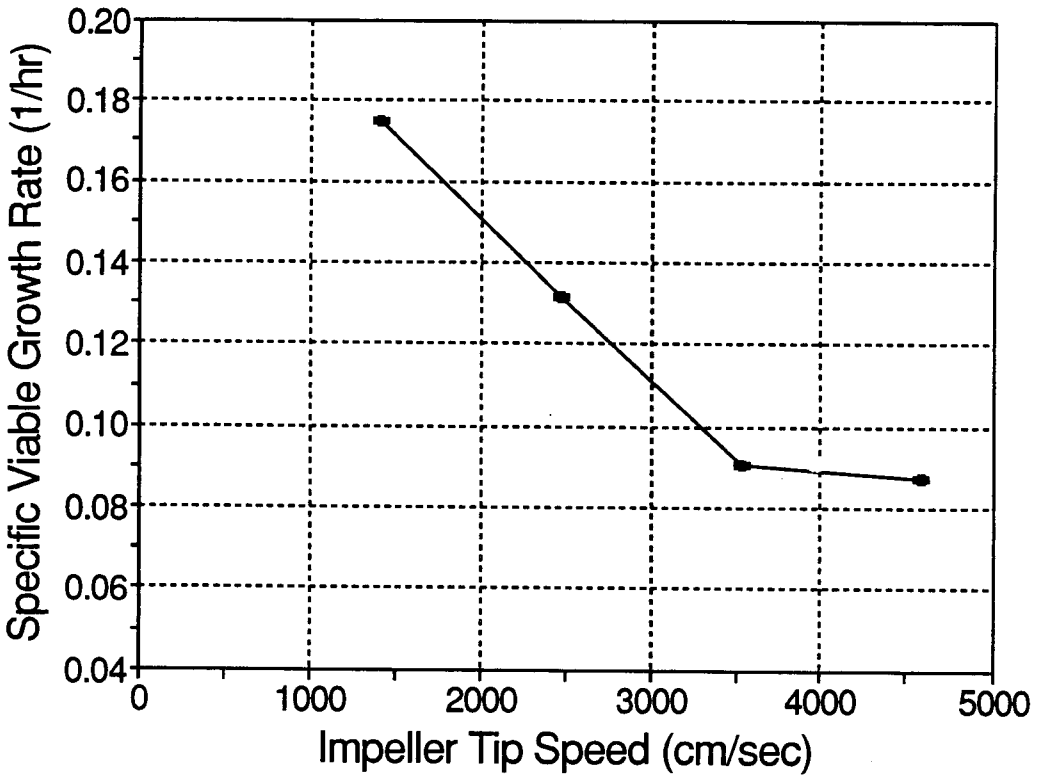

Figure 15. Specific viable growth rate (μ_v) vs. impeller tip speed.

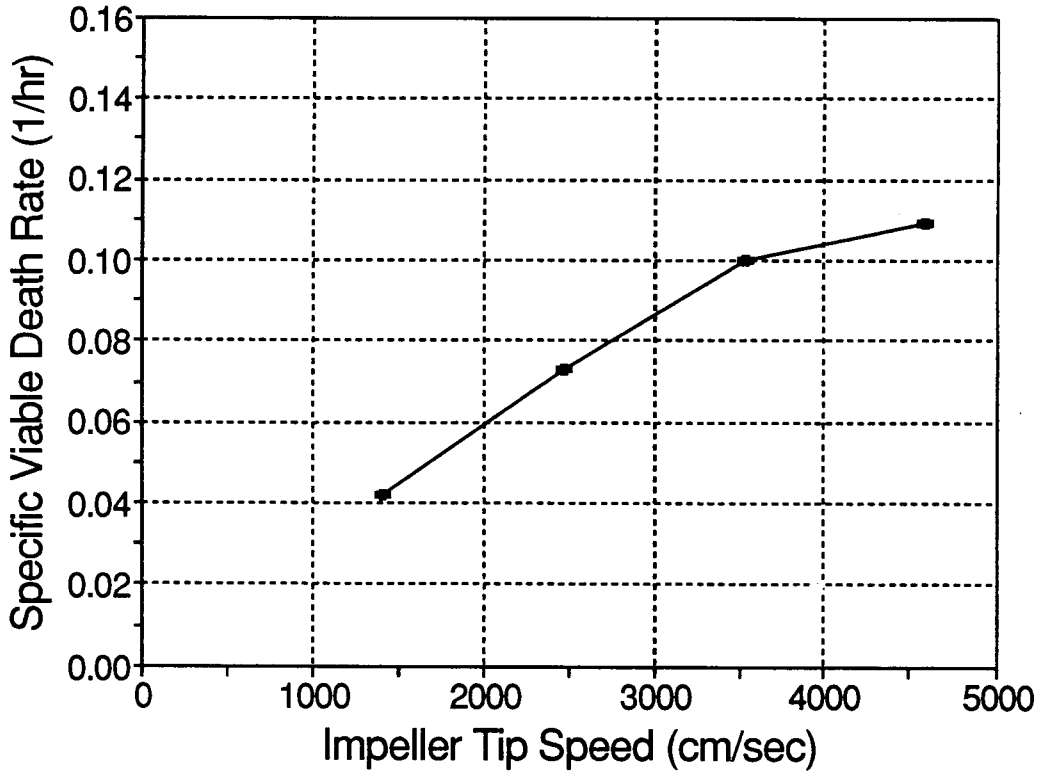

Figure 16. Specific viable death rate (k_d) vs. impeller tip speed.

Also, μ_v and k_d are correlated to initial k_La^0, a fundamental measure of both the impeller speed and aeration components of agitation intensity and a primary scale-up parameter for a stirred-tank bioreactor. Values for μ_v and k_d are plotted vs. k_La^0 in Figures 17 and 18. The initial k_La^0 is a function of the impeller speeds and is typically correlated to a power law eqution of the form (Roberts et al, 1992)

$$kLa = \alpha\,(rpm)^{\beta} \qquad [11]$$

where α and β are experimentally determined constants. From Figure 2, β was determined by the slope of a log-log plot of k_La vs. impeller speed. The linear regression output for β is 1.51 and is listed in Appendix B. When μ_v is correlated to initial k_La^0, the curve shows a power-law type of decreases with increasing k_La. Conservely, k_d shows a power law decay with increasing k_La.

In summary, in the stirred-tank impeller tip speed region of 1,414 cm/sec to 4,596 cm/sec (initial k_La of 4.75 hr^{-1} to 19.24 hr^{-1} at 0.425 vvm), hydrodynamic forces have a profound effect on culture growth. However, as we will see in the next section, oxygen mass transfer is not limiting to curve growth. Therefore, hydrodynamic shear forces are limiting biomass productivity in this region.

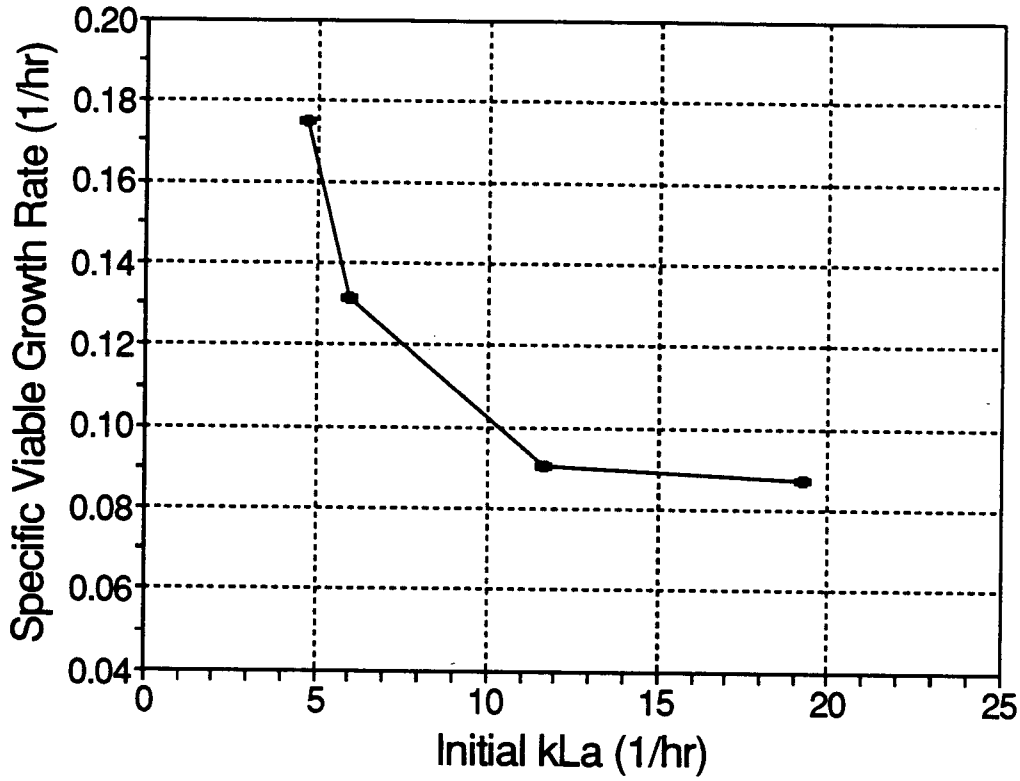

Figure 17. Specific viable growth rate (μ_v) vs. initial k_La.

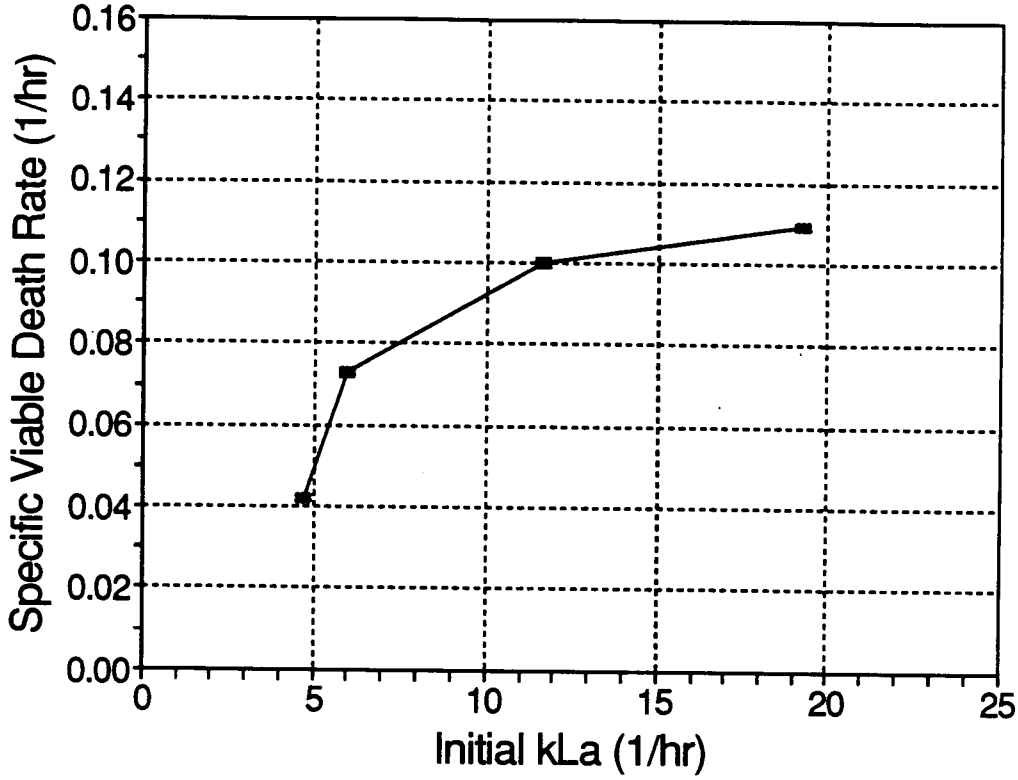

Figure 18. Specific viable death rate (k_d) vs. initial k_La.

pH

As shown in Figure 19 and 20, the pH value maintained stable between 5.2 and 5.5 during the entire cultivation period in the stirred-tank bioreactor at impeller speeds of 100-325 rpm and in shake flasks.

EFFECT OF MIXING SPEED ON OXYGEN MASS TRANSFER

D.O. Level vs Culture Time

The D.O. level for cultivation of cell cultures in stirred-tank bioreactor indicates if the oxygen supply to cell suspension liquid is sufficient. Payne et al [1987] reported that a oxygen level of 5 % of air saturation is the critical dissolved oxygen concentration. Below the critical D.O. concentration, the culture does not survive. In this present work, D.O. level (expressed as % of air saturation) was measured as a function of cultivation time at impeller speeds of 100-325 rpm, as shown in Figure 21. At 100 rpm and 325 rpm, only 4 and 9 of days of data were recorded respectively because the membrane on the tip of the D.O. probe failed.

In Figure 21, the initial D.O. level at the first hour of cultivation increased as impeller speed increased. At an impeller speed 325 rpm, the initial D.O. level was 92.5 % of air saturation. However, at impeller speed 100 rpm, the initial D.O. level was 72.0 % of air saturation. This is due to the improvement of oxygen mass transfer with increasing impeller speed.

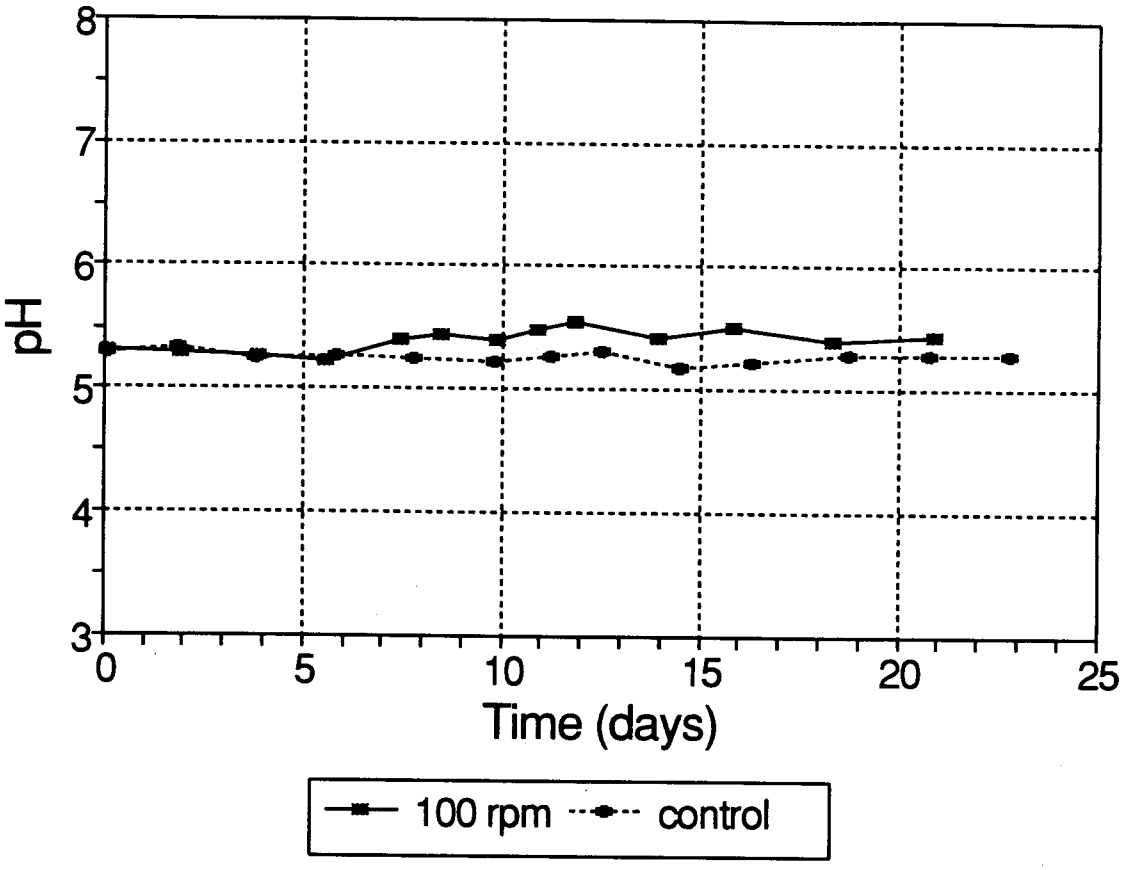

Figure 19. Culture pH vs. cultivation time at 100 rpm and in shake flasks.

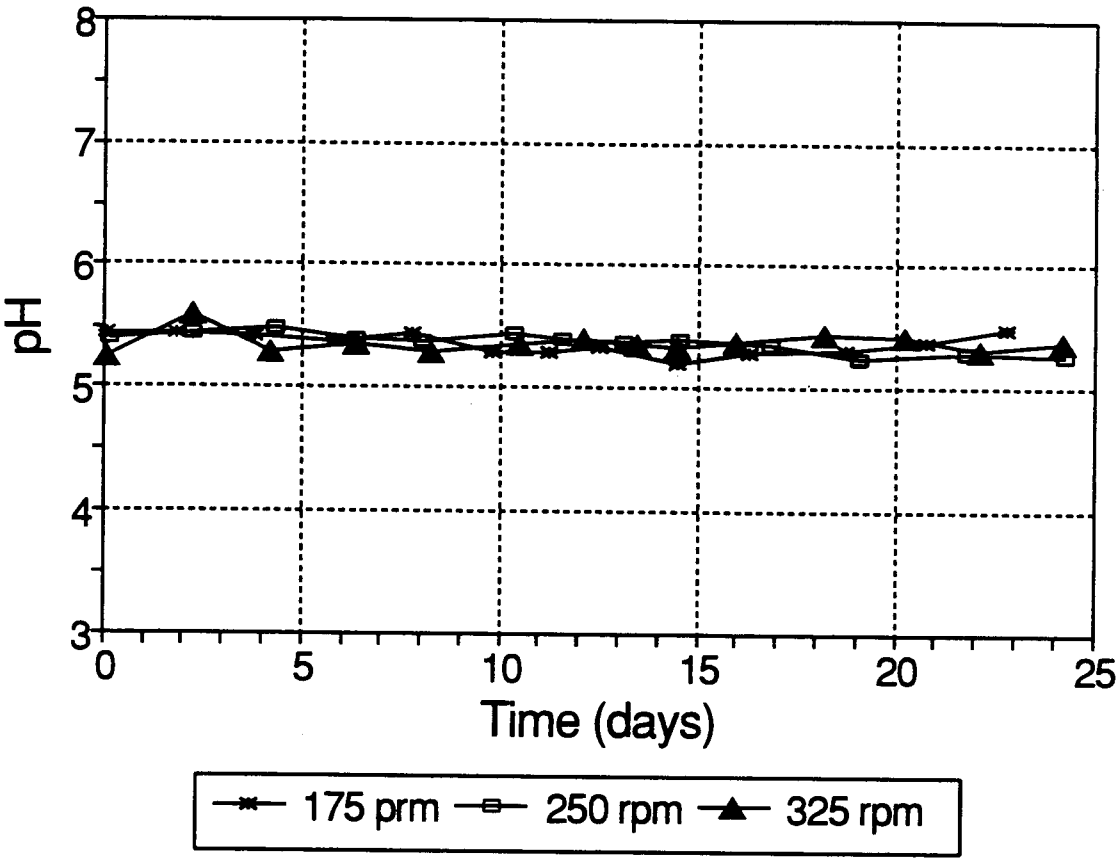

Figure 20. Culture pH vs. cultivation time at 175, 250, and 325 rpm.

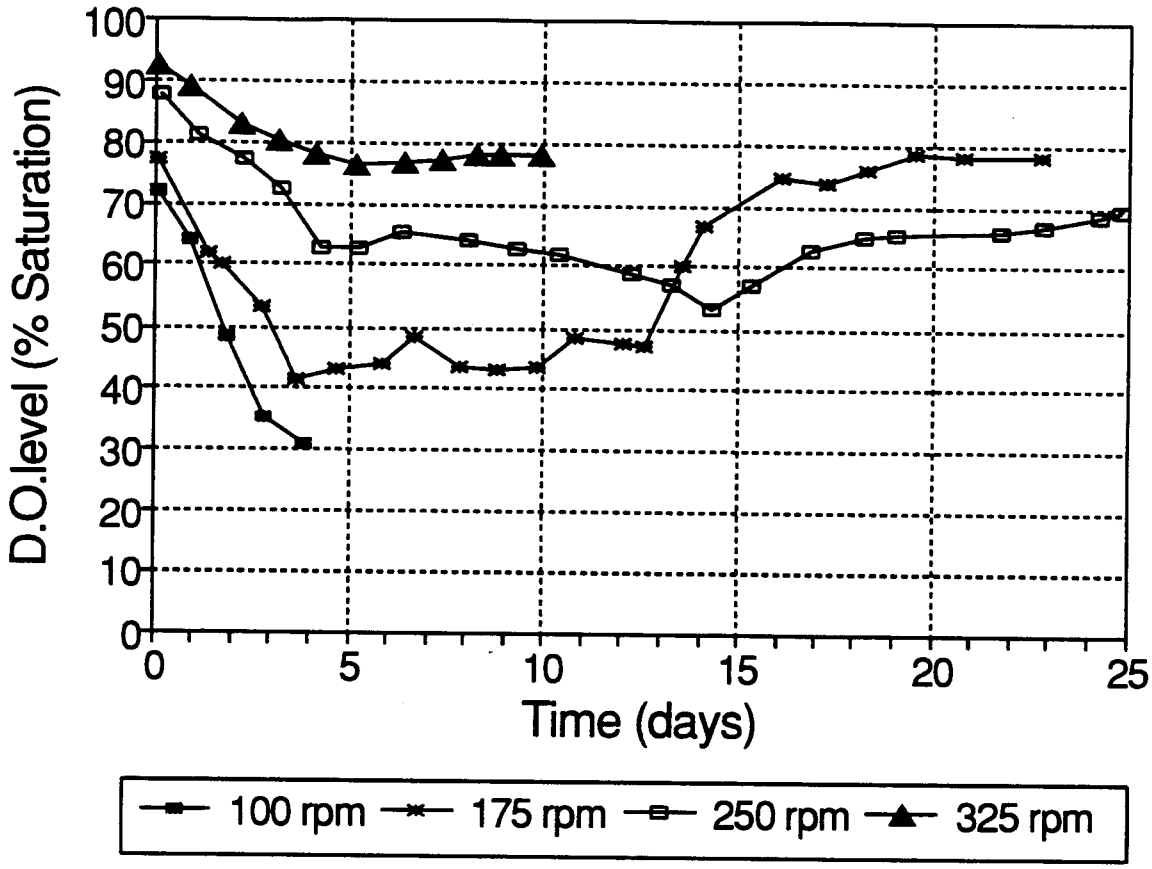

Figure 21. D.O. level vs. cultivation time at impeller speeds of 100 to 325 rpm.

This phenomenon will be discussed in more detail later in this section following presentation of Qo and k_La values in bioreactor culture.

Qo and k_La in Bioreactor Culture

Oxygen mass transfer to tobacco cell suspension cultures grown in a stirred-tank bioreactor to high cell density was characterized by measuring the volumetric oxygen mass transfer coefficient k_La in the cell suspension. Sample D.O. concentration vs. time data used for estimation of Qo and k_La in the culture by the dynamic method were already shown in Figure 3. Recall from Figure 3 that the data fitted model equations 1 and 2 very well, indicating that the dynamic method was suitable for estimation of Qo and k_La in the tobacco cell suspension culture. There were one limitation to this measurement technique. The D.O. level had to be at least 25 % of air saturation, so that the data obtained was always above 10 % of air saturation, the nominal critical D.O. level.

Respiration rate (Qo) vs. cultivation time data at impeller speeds of 100 to 325 rpm are shown in Figures 22 and 23. At 175 and 250 rpm, the Qo vs. time curve has two peaks. For example, at impeller speed 175 rpm, Qo increases rapidly during the first five days and then reaches the first peak. After reaching the first peak, Qo decreases slightly, and then slowly increases again to the second peak. When comparing the Qo and the D.O. level vs. time curves at 175 rpm, Qo increases while the D.O level goes down, and vice versa. Furthermore, when Qo reaches the second peak and then

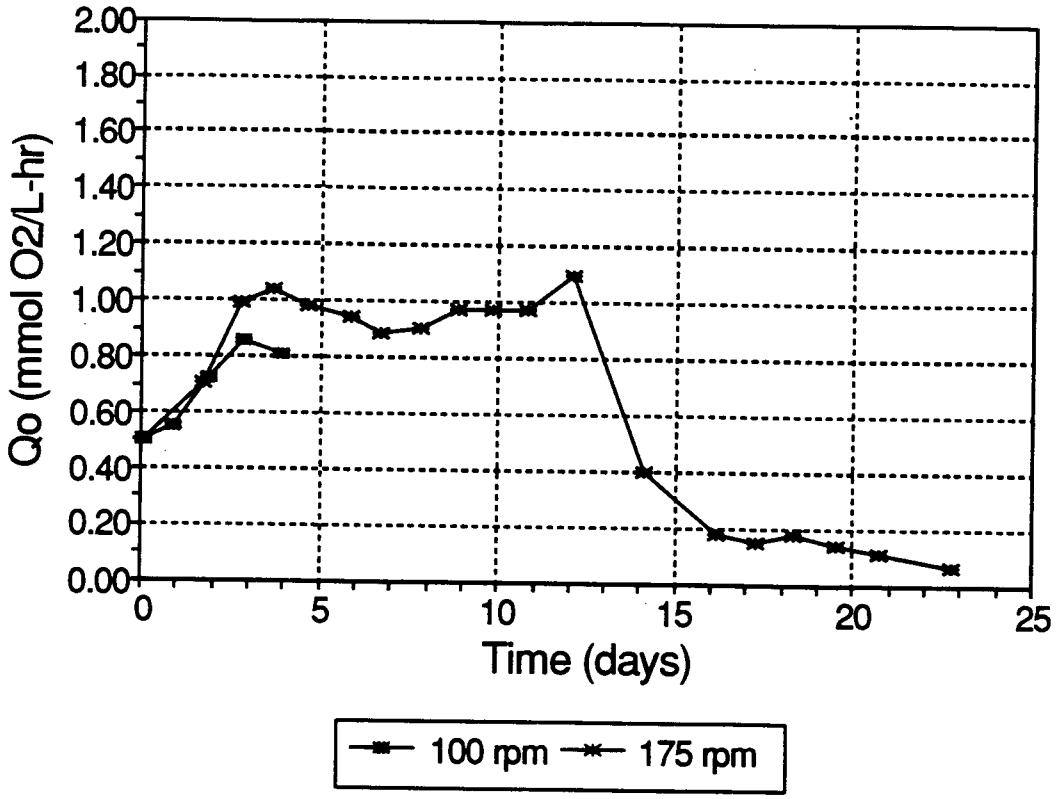

Figure 22. Culture Qo at 100 rpm and 175 rpm.

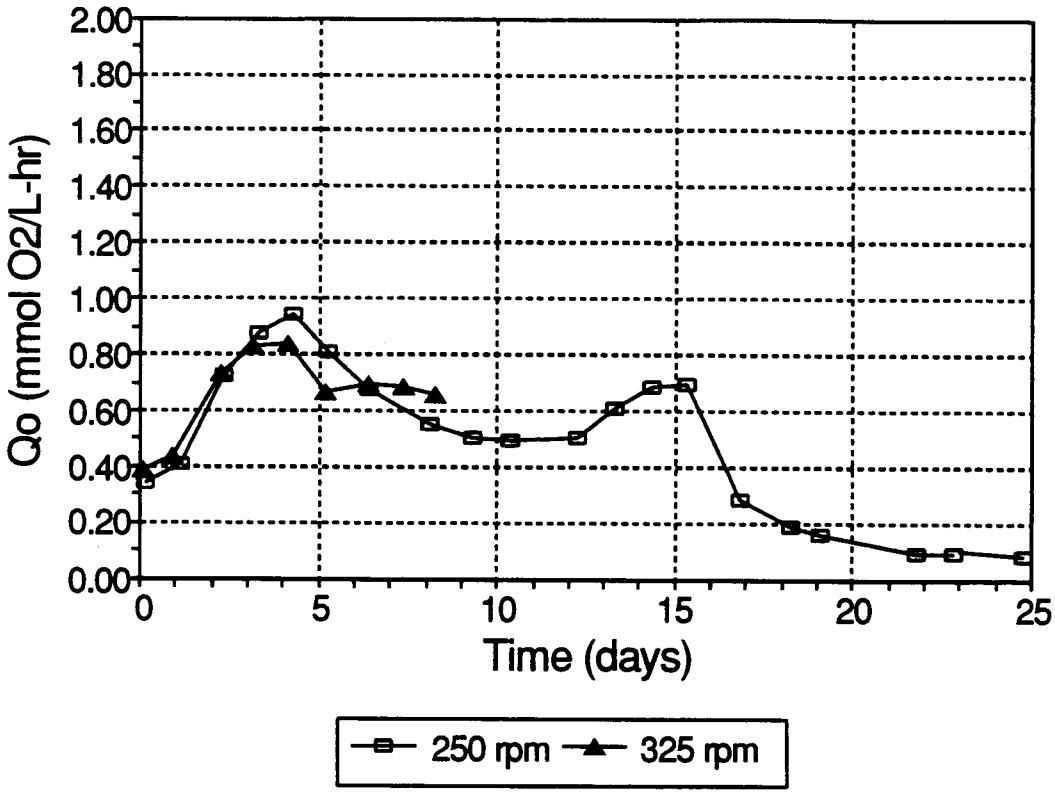

Figure 23. Culture Qo at 250 rpm and 325 rpm.

decreases, the D.O. level likewise increases. Concurrently, the viable biomass concentration starts to decrease at the same day. The same phenomenon is observed at 250 rpm.

Values for k_La in the bioreactor culture as a function of cultivation time for impeller speeds of 100 to 325 rpm are shown in Figure 24. During the early stages of cultivation, the cell density was relatively dilute and so k_La in the culture approximated the initial k_La^0. However, k_La in the culture decreased during the exponential phase of growth when the cell density was rapidly increasing. Thus the presence of the cells reduced the value of k_La. At 250 rpm, the k_La value at high cell density was around 7.5 hr^{-1}, versus 11.7 hr^{-1} after the first hour cultivation, a reduction of about 65%. At 175 rpm, the k_La value decreased from 8.9 hr^{-1} to 7.2 hr^{-1} during the first 4 days of culture and then was constant for the remainder of the cultivation period. This k_La value at high cell density was about 75 % of the k_La value of the first hour cultivation. These results are consistent with mass transfer studies of Ballica and Ryu (1993), who showed that k_La in airlift bioreactor culture of *D. stramonium* cells in high cell density was about 60 % of the initial k_La value. However, in Ballica and Ryu's work, the k_La values in airlift cell culture were determined by adding fresh biomass at successively higher loadings to the bioreactor, whereas in the present study the k_La values were obtained from the same culture over an entire batch cultivation period.

In summary, it appears that the presence of the cells reduces the oxygen mass transfer rate. However, over the range of impeller speeds of 175 to 325 rpm, the reduction in oxygen mass transfer

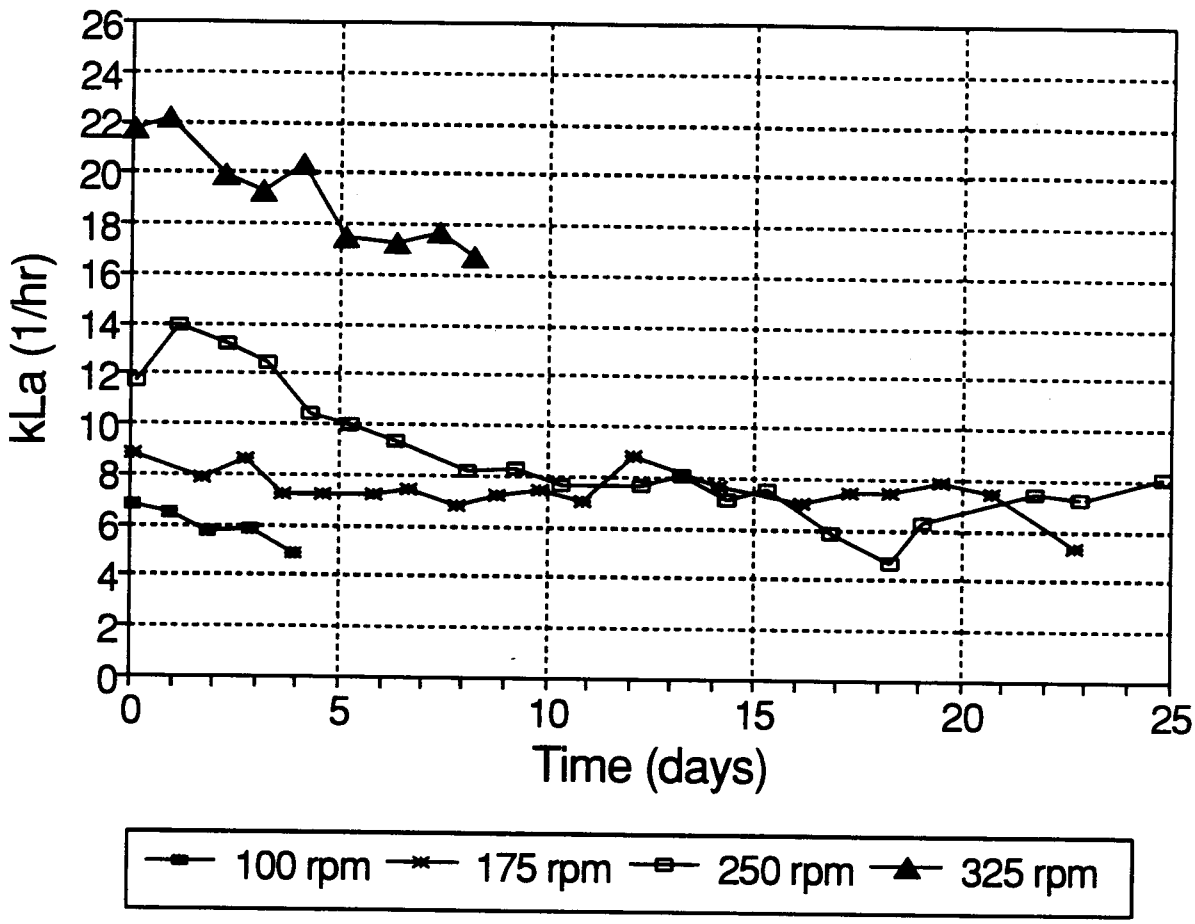

Figure 24. Culture k_La at impeller speeds of 100 to 325 rpm.

rate was not sufficient to cause oxygen starvation at any time during the cultivation.

Effect of Qo and k_La on the D.O. level vs Time Curve

The culture D.O. level is constantly changing over the 24 day cultivation period because the oxygen demand and oxygen mass transfer coefficients are changing as the biomass builds up within the culture. However, in a given short time period (one hour or so), the D.O. level is relatively constant and can be represented by a pseudo steady state given by

$$C_A = C_A^* - \frac{Qo}{k_La} \qquad [12]$$

Thus, Qo and k_La vs. culture time curves set the value of the D.O. concentration as the cultivation proceeds. For example, at the impeller speed 175 rpm, the D.O. level decreased during the first 4 days while Qo increased and k_La decreased. The D.O. level then maintained steady for several days, and Qo and k_La also remained relatively constant. After day 13, the D.O. level started to increase while Qo decreased. However, k_La was still constant. Finally, after day 19, the D.O. level rose back to the D.O. level at the first day of cultivation. The same phenomenon was observed at the other impeller speeds, implying that the D.O. level can be predicted by Qo and k_La when the pseudo steady state assumption is valid.

SUMMARY AND CONCLUSION

Suspension cultures of *Nicotiana tabacum* (tobacco) cells were cultivated in a 3 L stirred-tank bioreactor to determine the effect of impeller speed on cell damage and oxygen mass transfer rates during a 25 day batch cultivation period.

The final cell density and the number fraction of viable cells decreased as the impeller speed increased from 100 to 325 rpm. To further assess the effect of impeller speed on culture growth, the specific viable cell growth rate (μ_v) in exponential growth phase and the specific viable cell death rate (k_d) in stationary growth rate were determined at impeller speeds of 100, 175, 250, and 325 rpm. As the impeller speed increased, the value of μ_v decreased rapidly between 100 rpm and 250 rpm, and then more slowly from 250 to 325 rpm. The value of k_d increased rapidly between 100 rpm and 250 rpm, and then more slowly from 250 to 325 rpm. These results implied that *N. tabacum* cells were sensitive to hydrodynamic shear forces at impeller speeds of 100 to 325 rpm.

The specific cell death rate k_d quantified the loss in viable cell density due to hydrodynamic shear force during the stationary phase. The result showed that k_d for low-shear flask culture was lower than k_d for bioreactor cultivation at impeller speeds greater than 100 rpm.

The tip speed Reynolds number and initial k_La^0 were also correlated to μ_v and k_d. The results showed that μ_v decreased and k_d increased between impeller tip number of 1414 and 4596 cm/sec (initial k_La^0 4.8 hr^{-1} and 19.2 hr^{-1} at 0.425 vvm).

The value of k_La and Qo in bioreactor culture were measured at two-day intervals during the cultivation period by the dynamic method. The respiration rate (Qo) vs. cultivation time curve showed two peaks at impeller speeds of 175 and 250 rpm. After reaching the second peak, Qo rapidly decreased. Concurrently, the viable biomass concentration started to decrease, and stationary phase of growth was also achieved.

Values of k_La during the early stages of cultivation in the bioreactor culture approximated the initial k_La^0, due to the relatively dilute cell density (2g DCW/L) in the liquid medium. However, the value of k_La decreased when the cell density increased. Thus, the presence of the cells reduced the oxygen mass transfer rate. However, over the range of impeller speeds of 175 to 325 rpm, the reduction in oxygen mass transfer rate was not sufficient to cause oxygen starvation at any time during the cultivation.

Therefore, at an aeration rate of 0.425 vvm, the cell growth is affected by hydrodynamic stress and not by oxygen mass transfer rates at impeller speeds of 100 to 325 rpm.

In summary, for bioreactor scale up use, the optimal growth rate and the maximum cell density could be obtained at the low impeller speeds and with a suitable aeration rate.

RECOMMENDATIONS

In this present work, the viable death rate k_d increased as impeller speed increased. However, it is not known if the viable cell growth rate is higher at impeller speeds less than 100 rpm. It is possible that an optimal growth rate could exist at impeller speeds less than 100 rpm.

In the further work, the impeller speed could be set at 70 and 130 rpm to find the optimal growth rate at the fixed aeration rate 0.425 vvm. It is possible that this aeration rate can still provide suitable oxygen transfer in the bioreactor even at these low agitation speeds.

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APPENDICES

APPENDIX A: PROTOCOLS

Inoculation and Sampling of the 3 L Applikon Stirred-Tank Bioreactor for Plant Cell Culture

Reactor Cleaning and Sterilization

1. Use clean hot water at high flow rate to flush remaining dead from assembled bioreactor about 10 minutes (at least ten minutes is needed to help separate the headplate and the glass vessel).

2. Wash glass and stainless steel parts of the reactor with hot water, and then use liquid detergent to clean all parts. Never use scouring material to clean the metal parts.

3. After cleaning, dry the parts in air and reassemble the reactor. Take care not to damage or forget any O-rings, since this can cause contamination during the next run.

4. Close all tubings with clamps between the headplate and the filter except one air outlet tubing to maintain pressure equilibrium in and outside the reactor.

5. Close all open tubing ends with cotton and cover the end with sterizable foil or paper except that air outlet tubing mentioned in step 4.

6. Fill the reactor with 1.6 L of distilled water and autoclave (121 °C, 15 psig), on slow exhaust, for 50 minutes.

7. Inspect the reactor assembly for any leaks. Leaks may occur because of: loose tubing, punctures in the tubing, loose fittings, or a filter that came off during autoclaving. If leaks are present, fix them.

8. Cool down the reactor for two hours after autoclaving and pour out the distilled water.

9. Refill the reactor with 1.6 L medium (formula found in Table 1) and plug the D.O. probe into one port on the headplate.

10. follow the steps of 4 and 5 again and autoclave reactor (121 °C, 15 psig), on slow exhaust, for one hour. Also, autoclave a foil covered glass funnel (needed for inoculation).

11. After autoclaving, cool down the reactor in the laminar hood to room temperature.

Inoculation: (all procedures are carried out in the laminar hood)

12. Replace the electrolyte in the D.O. probe until there are no bubbles coming out from the vent tube (please refer the manual instruction of Virtis D.O. probe and meter).

13. Wash all exposed parts surrounding the inoculated port on the headplate of the reactor with a 95% ethanol solution.

14. Remove the M 18x1.5 plug from its port, being careful not to touch any surfaces. Hold the plug with one hand, and with the other hand insert the autoclaved, flame-sterilized funnel into the port using flame-sterilized forceps.

15. Obtain three 500 mL flasks containing 150 mL of 9 day old tobacco cell suspension cultures, Pour each flask into the reactor through the funnel. Prior to pouring out the contents in the 500 mL flask, flame the lip of the flask.

16. Remove the funnel and screw in the inoculation port until completed sealed.

Sampling:

17. Autoclave one sample vial (121 ^{o}C, 15 psig), on fast exhaust, for 10 minutes.

18. Open the flexible hose on sampling line and take a 25 mL sample from the cell culture into the sample vial with a 60 mL syringe.

19. Clamp flexible hose immediately, unscrew the sample vial, and use 95% ethanol washing the surrounding of the sample cap immediately.
20. Screw the sample vial immediately.

Microscopic analysis of number fraction of viable and non viable cells after sampling

1. Mix 0.5 mL culture sample with a drop of Evan's blue stain (0.25 wt%).

2. Do not let the sample sit.

3. Immediately, load the stained culture sample into one well of a Fuchs-Rosenthal hemocytometer (1/16 mm^2 per square and 2/10 mm deep) with a Pastuer pipet.

4. Count the number of intact cells, ruptured cells, and cell aggregates on 16 triple ruled squares ($1 mm^2$).

5. Dead and ruptured cells show blue color, and intact cells have light blue or transparent color.

D.O. electrode maintenance

1. The membrane should be examined routinely after each fermentation cycle and replaced if any deterioration is evident.

2. The membrane, membrane cap, and electrode should be cleaned with small amount of electrolyte to flush out the inpure residue suspension after each fermentation cycle.

3. After the electrode is used for a period of time (3 - 4 cycles), inpure residue may form on the cathode and anode. It will alter the electrode performance. Steps to clean the anode and cathode can be found in the instruction manual of D.O. electrode, the section of maintenance.

APPENDIX B: TABULAR DATA

Table B-1. Viable growth rate vs impeller speed.

Impeller Speed (rpm)	Viable Growth Rate (1/hr)
100	0.175
175	0.132
250	0.091
325	0.087
Shake Flask	0.097

Table B-2. Viable death rate vs impeller speed.

Impeller Speed (rpm)	Viable Death Rate (1/day)
100	0.042
175	0.073
250	0.100
325	0.109
Shake Flask	0.036

Table B-3. Viable growth rate vs impeller tip speed.

Impeller Tip Speed (cm/sec)	Viable Growth Rate (1/hr)
1414	0.175
2475	0.132
3535	0.091
4596	0.087
Shake Flask	0.097

Table B-4. Viable death rate vs impeller tip speed.

Impeller Tip Speed (cm/sec)	Viable Death Rate (1/day)
1414	0.042
2475	0.073
3535	0.100
4596	0.109
Shake Flask	0.036

Table B-5. Viable growth rate vs initial kLa.

Initial kLa (1/hr)	Viable Growth Rate (1/day)
4.75	0.175
5.92	0.132
11.67	0.091
19.24	0.087

Table B-6. Viable death rate vs initial kLa.

Initial kla (1/hr)	Viable Death rate (1/day)
4.75	0.042
5.92	0.073
11.67	0.100
19.24	0.109

Table B-7-1. Initial kLa vs impeller speed at 0.43 vvm

Impeller Speed (rpm)	Initial kLa (1/hr)		
	trial 1	trial 2	average
100	4.7	4.8	4.8
175	5.7	6.1	5.9
250	11.4	12.0	11.7
325	18.8	19.7	19.2
400	44.4	37.8	41.1

Table B-7-2. Regression output for Beta.

Constant		-5.676
Std Err of Y Est		0.350
R Squared		0.881
No. of Observations		5
Degrees of Freedom		3
Beta:	1.509	
Std Err of Coef.	0.320	

Table B-8. Bioreactor data for Run# 12 at 100 rpm.

Impeller Speed:	100	rpm
Aeration Rate:	850	mL/min
Total Culture Volume	2050	mL
Run #:	12	
Start Date:	10/25/93	

Time (hr)	Time (day)	Total Cell Density, X (g DCW/L)	pH	Cell Viability % Viable	% Nonviable	Viable Cell Density, Xv (g/L)
1.0	0.04	2.44	5.32	88.0	12.0	2.15
45.0	1.88	4.48	5.28	82.6	17.4	3.70
93.5	3.90	4.59	5.27	80.1	19.9	3.68
133.0	5.54	8.58	5.23	81.8	18.2	7.02
179.0	7.46	10.66	5.40	74.5	25.5	7.94
203.0	8.46	9.93	5.44	72.8	27.2	7.23
237.0	9.88	10.31	5.40	72.8	27.2	7.51
261.0	10.88	11.83	5.50	69.3	30.7	8.20
285.0	11.88	9.86	5.55	70.0	30.0	6.90
334.0	13.92	9.96	5.43	69.4	30.6	6.91
380.0	15.83	9.05	5.51	68.1	31.9	6.16
441.0	18.38	8.52	5.39	64.9	35.1	5.53
502.0	20.92	8.92	5.44	57.6	42.4	5.14

Table B-9. Bioreactor data for Run# 12 at 100 rpm (continued).

Impeller Speed:	100	rpm
Aeration Rate:	850	mL/min
Total Culture Volume	2050	mL
Run #:	12	
Start Date:	10/25/93	

Time (hr)	Time (day)	D.O. level % Saturation	Qo (mmol O2/L-hr)	kLa (1/hr)
1.0	0.04	72.0	0.5	6.83
22.0	0.92	64.0	0.552	6.51
45.0	1.88	48.4	0.724	5.82
68.5	2.85	35.5	0.855	5.87
93.5	3.90	31.1	0.808	4.90
143.0	5.96	39.7	1.171	8.36
165.0	6.88	40.6	0.847	5.83
188.0	7.83	36.0	0.985	6.41
212.0	8.83	25.2	1.196	6.21
237.0	9.88	24.6	1.242	6.17
261.0	10.88	22.0	1.172	6.09
285.0	11.88	28.6	1.005	7.30
334.0	13.92	46.0	0.489	4.88
355.0	14.79	48.9	0.411	4.52
380.0	15.83	55.2	0.405	5.34
404.0	16.83	54.6	0.259	3.33
441.0	18.38	55.7	0.159	3.15
505.0	21.04	58.3	0.112	2.59
525.0	21.88	58.0	0.072	2.62
597.0	24.88	56.0	0.064	1.71

Table B-10. Bioreactor data for Run# 13 at 175 rpm.

Impeller Speed:	175	rpm
Aeration Rate:	850	mL/min
Total Culture Volume	2050	mL
Run #:	13	
Start Date:	12/02/93	

Time (hr)	Time (day)	Total Cell Density, X (g DCW/L)	pH	Cell Viability % Viable	% Nonviable	Viable Cell Density, Xv (g/L)
1.0	0.04	2.69	5.44	84.6	15.4	2.28
44.0	1.83	4.37	5.45	80.9	19.1	3.54
91.0	3.79	5.41	5.42	78.3	21.7	4.24
140.0	5.83	7.77	5.37	70.9	29.1	5.51
188.0	7.83	8.79	5.44	75.6	24.4	6.65
236.0	9.83	8.89	5.30	70.3	29.7	6.25
270.0	11.25	10.00	5.28	65.4	34.6	6.54
300.0	12.50	11.01	5.33	62.0	38.0	6.83
347.0	14.46	10.47	5.20	64.6	35.4	6.76
392.0	16.33	9.12	5.30	62.1	37.9	5.66
449.0	18.71	7.70	5.32	61.2	38.8	4.71
499.0	20.79	7.20	5.38	52.7	47.3	3.79
547.0	22.79	7.43	5.49	46.8	53.2	3.48

Table B-11. Bioreactor data for Run# 13 at 175 rpm (continued).

Impeller Speed:	175	rpm
Aeration Rate:	850	mL/min
Total Culture Volume	2050	mL
Run #:	13	
Start Date:	12/02/93	

Time (hr)	Time (day)	Qo (mmol O2/L-hr	kLa (1/hr)
1.0	0.04	0.500	8.85
42.0	1.75	0.702	7.86
66.0	2.75	0.990	8.61
88.5	3.69	1.044	7.23
111.5	4.65	0.981	7.23
140.0	5.83	0.950	7.22
160.0	6.67	0.884	7.48
188.0	7.83	0.902	6.82
212.0	8.83	0.969	7.28
236.0	9.83	0.979	7.47
260.0	10.83	0.975	7.02
290.0	12.08	1.096	8.88
339.0	14.13	0.402	7.64
387.0	16.13	0.177	7.05
415.0	17.29	0.146	7.48
439.0	18.29	0.179	7.45
468.0	19.50	0.145	7.90
498.0	20.75	0.115	7.42
546.0	22.75	0.063	5.35

Table B-12. Bioreactor data for Run# 13 at 175 rpm (continued).

Impeller Speed:	175	rpm
Aeration Rate:	850	mL/min
Total Culture Volume	2050	mL
Run #:	13	
Start Date:	12/02/93	

Time (hr)	time (day)	D.O. level % Saturation
1.0	0.04	77.4
34.0	1.42	62.1
42.0	1.75	60.2
66.0	2.75	53.1
88.5	3.69	41.3
111.5	4.65	43.2
140.0	5.83	44.1
160.0	6.67	48.6
188.0	7.83	43.5
212.0	8.83	43.1
236.0	9.83	43.4
260.0	10.83	48.5
290.0	12.08	47.7
302.0	12.58	47.2
326.0	13.58	60.0
339.0	14.13	66.8
387.0	16.13	74.4
415.0	17.29	73.7
439.0	18.29	75.8
468.0	19.50	78.6
498.0	20.75	78.2
546.0	22.75	78.3

Table B-13. Bioreactor data for Run# 11 at 250 rpm.

Impeller Speed:	250	rpm
Aeration Rate:	850	mL/min
Total Culture Volume	2050	mL
Run #:	11	
Start Date:	09/24/93	

Time (hr)	Time (day)	Total Cell Density, X (g DCW/L)	pH	Cell Viability % Viable	% Nonviable	Viable Cell Density, Xv (g/L)
2.5	0.10	2.46	5.40	74.1	25.9	1.82
53.5	2.23	3.01	5.45	68.2	31.8	2.05
103.0	4.29	4.78	5.50	65.7	34.3	3.14
152.5	6.35	4.25	5.40	68.6	31.4	2.92
194.5	8.10	4.91	5.38	65.6	34.4	3.22
248.5	10.35	7.90	5.45	60.0	40.0	4.74
278.5	11.60	9.21	5.40	58.8	41.2	5.42
315.0	13.13	8.82	5.38	60.0	40.0	5.29
349.5	14.56	9.82	5.40	57.1	42.9	5.61
404.5	16.85	8.98	5.35	51.0	49.0	4.58
457.5	19.06	7.90	5.25	46.4	53.6	3.67
522.5	21.77	6.50	5.28	39.5	60.5	2.57
582.5	24.27	6.83	5.26	32.4	67.6	2.21

Table B-14. Bioreactor data for Run# 11 at 250 rpm (continued).

Impeller Speed:	250	rpm
Aeration Rate:	850	mL/min
Total Culture Volume	2050	mL
Run #:	11	
Start Date:	09/24//93	

Time (hr)	Time (day)	D.O. level % Saturation	Qo (mmol O2/L-hr)	kLa (1/hr)
2.5	0.10	88.0	0.341	11.70
27.0	1.13	81.0	0.411	13.93
55.0	2.29	77.0	0.718	13.24
78.5	3.27	72.4	0.883	12.50
103.0	4.29	63.0	0.940	10.48
126.0	5.25	63.0	0.813	10.02
152.5	6.35	65.6	0.680	9.43
194.0	8.08	64.0	0.549	8.23
223.0	9.29	63.0	0.501	8.29
248.5	10.35	62.0	0.492	7.67
294.5	12.27	59.0	0.508	7.62
318.5	13.27	57.2	0.613	8.12
343.5	14.31	53.4	0.682	7.16
368.0	15.33	56.9	0.693	7.53
404.5	16.85	63.0	0.289	5.87
438.5	18.27	65.0	0.186	4.74
457.5	19.06	65.4	0.154	6.22
522.5	21.77	66.0	0.090	7.45
548.5	22.85	67.0	0.099	7.25
582.5	24.27	68.8	---	---
595.5	24.81	69.3	0.085	8.12

Table B-15. Bioreactor data for Run# 14 at 325 rpm.

Impeller Speed:	325	rpm
Aeration Rate:	850	mL/min
Total Culture Volume	2050	mL
Run #:	14	
Start Date:	01/18/94	

Time (hr)	Time (day)	Total Cell Density, X (g DCW/L)	pH	Cell Viability % Viable	% Nonviable	Viable Cell Density, Xv (g/L)
2.0	0.08	2.75	5.24	66.5	33.5	1.83
54.0	2.25	4.22	5.60	66.4	33.6	2.80
100.0	4.17	3.49	5.30	58.4	41.6	2.04
153.0	6.38	6.78	5.35	54.5	45.5	3.70
198.0	8.25	6.78	5.30	56.8	43.2	3.85
251.0	10.46	7.68	5.35	52.9	47.1	4.06
291.0	12.13	8.63	5.39	50.6	49.4	4.37
323.0	13.46	8.50	5.35	46.0	54.0	3.91
347.0	14.46	8.85	5.33	49.6	50.4	4.39
384.0	16.00	7.28	5.37	47.0	53.0	3.42
437.0	18.21	6.10	5.45	42.3	57.7	2.58
485.0	20.21	5.64	5.42	41.9	58.1	2.36
531.0	22.13	4.91	5.32	36.7	63.3	1.80
582.0	24.25	4.87	5.38	29.5	70.5	1.44

Table B-16. Bioreactor data for Run# 14 at 325 rpm (continued).

Impeller Speed:	325	rpm
Aeration Rate:	850	mL/min
Total Culture Volume	2050	mL
Run #:	14	
Start Date:	01/18/94	

Time (hr)	Time (day)	Qo (mmol O2/L-hr	kLa (1/hr)
2.0	0.08	0.389	21.70
21.0	0.88	0.434	22.19
54.0	2.25	0.734	19.88
76.0	3.17	0.827	19.33
99.0	4.13	0.842	20.37
123.0	5.13	0.672	17.48
153.0	6.38	0.698	17.23
177.0	7.38	0.689	17.73
198.0	8.25	0.658	16.76

Time (hr)	time (day)	D.O. level % Saturation
2.0	0.08	92.5
21.0	0.88	89.1
54.0	2.25	83.1
76.0	3.17	80.3
99.0	4.13	78.3
123.0	5.13	76.3
153.0	6.38	76.9
177.0	7.38	77.0
198.0	8.25	78.3
213.0	8.88	78.2
237.0	9.88	78.3

Table B-17. Shake Flask data in orbital shaker at 150 rpm.

Flask Volume:	125	mL
Shaker Speed:	150	rpm
Total Culture Volume	45	mL
Run #:	13	
Start Date:	12/02/93	

Time (hr)	Time (day)	Total Cell Density, X (g DCW/L)	pH	Cell Viability % Viable	% Nonviable	Viable Cell Density, Xv (g/L)
1.0	0.04	2.52	5.30	95.5	4.5	2.41
44.0	1.83	3.66	5.33	93.6	6.4	3.43
91.0	3.79	5.42	5.24	80.2	19.8	4.35
140.0	5.83	8.94	5.27	73.5	26.5	6.57
188.0	7.83	7.86	5.24	80.8	19.2	6.35
236.0	9.83	8.70	5.23	77.4	22.6	6.73
270.0	11.25	10.34	5.27	76.4	23.6	7.90
300.0	12.50	12.02	5.32	77.4	22.6	9.30
347.0	14.46	12.87	5.19	75.2	24.8	9.68
392.0	16.33	11.34	5.22	75.8	24.2	8.60
449.0	18.71	10.81	5.30	79.5	20.5	8.59
499.0	20.79	10.60	5.28	70.1	29.9	7.43
547.0	22.79	10.34	5.30	68.5	31.5	7.08

Table B-18. Regression output of viable growth rate. and death rate at 100 rpm.

Constant		0.822
Std Err of Y Est		0.180
R Squared		0.915
No. of Observations		5
Degrees of Freedom		3
viable growth rate:	0.175	1/day
Std Err of Coef.	0.031	

Constant		2.505
Std Err of Y Est		0.049
R Squared		0.933
No. of Observations		6
Degrees of Freedom		4
viable death rate	-0.042	1/day
Std Err of Coef.	0.006	

Table B-19. Regression output of viable growth rate. and death rate at 175 rpm.

Constant		0.917
Std Err of Y Est		0.091
R Squared		0.964
No. of Observations		5
Degrees of Freedom		3
viable growth rate	0.132	1/day
Std Err of Coef.	0.015	

Constant		2.898
Std Err of Y Est		0.056
R Squared		0.970
No. of Observations		6
Degrees of Freedom		4
viable death rate	-0.073	1/day
Std Err of Coef.	0.006	

Table B-20. Regression output of viable growth rate. and death rate at 250 rpm.

Constant		0.577
Std Err of Y Est		0.117
R Squared		0.928
No. of Observations		7
Degrees of Freedom		5
viable growth rate	0.091	1/day
Std Err of Coef.	0.011	

Constant		3.193
Std Err of Y Est		0.046
R Squared		0.989
No. of Observations		5
Degrees of Freedom		3
viable death rate	-0.100	1/day
Std Err of Coef.	0.006	

Table B-21. Regression output of viable growth rate and death rate at 325 rpm.

Constant		0.631
Std Err of Y Est		0.213
R Squared		0.701
No. of Observations		5
Degrees of Freedom		3
viable growth rate:	0.087	1/day
Std Err of Coef.	0.033	

Constant		3.011
Std Err of Y Est		0.055
R Squared		0.986
No. of Observations		6
Degrees of Freedom		4
viable death rate	-0.109	1/day
Std Err of Coef.	0.007	

Table B-22. Regression output of viable growth rate and death rate in shake flask cultivation.

Constant		1.048
Std Err of Y Est		0.143
R Squared		0.916
No. of Observations		8
Degrees of Freedom		6
viable growth rate	0.097	1/day
Std Err of Coef.	0.012	

Constant		2.781
Std Err of Y Est		0.038
R Squared		0.930
No. of Observations		5
Degrees of Freedom		3
viable death rate:	-0.036	1/day
Std Err of Coef.	0.006	