University of Iowa
Iowa Research Online

Spring 2010

Mechanism of CO2 inhibition in insect cell culture

Sucheta Gowthami Vajrala
University of Iowa

MECHANISM OF CO_2 INHIBITION IN INSECT CELL CULTURE

by

Sucheta Gowthami Vajrala

A thesis submitted in partial fulfillment
of the requirements for the Master of Science degree
in Chemical and Biochemical Engineering
in the Graduate College of
The University of Iowa

May 2010

Thesis Supervisor: Professor David W. Murhammer

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

_____

MASTER'S THESIS

_____

This is to certify that the Master's thesis of

Sucheta Gowthami Vajrala

has been approved by the Examining Committee
for the thesis requirement for the Master of Science
degree in Chemical and Biochemical Engineering
at the May 2010 graduation.

Thesis Committee: _____
David W. Murhammer, Thesis Supervisor

_____
Mani Subramanian

_____
Tonya Peeples

To my parents, whose support and encouragement I will never forget

ACKNOWLEDGMENTS

ABSTRACT

The prominence of insect cell culture has grown rapidly due to its ability to produce baculovirus biopesticides and recombinant proteins using the Baculovirus Expression Vector System. A critical problem in the mass production of these products is CO_2 accumulation to inhibitory levels within the bioreactor. The current research investigated the effect of elevated CO_2 concentrations on insect cell growth and metabolism and the roles of oxidative stress and intracellular pH (pH_i) in CO_2 inhibition.

Spodoptera frugiperda Sf-9 insect cells were cultured in a 3 L bioreactor (1.2 L working volume) controlled at 20% air saturation, 27^oC and a pH of 6.2. The cells were exposed to a constant CO_2 concentration by purging the medium with CO_2 and the headspace with air. The experiments were repeated for different CO_2 concentrations and samples were taken every 24 h to determine cell density, viability, metabolism and oxidative stress.

The population doubling time (PDT) of Sf-9 cells increased with increasing CO_2 concentration. Specifically, the PDT for 0-37, 73, 147, 183 and 220 mm Hg CO_2 concentrations were 23.2 ± 6.7, 32.4 ± 7.2, 38.1 ± 13.3, 42.9 ± 5.4 and 69.3 ± 35.9 h (n = 3 or 4; 95% confidence level), respectively. An 80 mL working volume shaker flask was maintained as a control and had an average PDT of 24.9 ± 3.1 h (n = 7; 95% confidence level). The viability of cells in all experiments was above 90%. The osmolality for all bioreactor experiments was observed to be 300 to 360 mOsm/kg, a range that is known to have a negligible effect on insect cell culture. Elevated CO_2 concentration did not alter the cell specific glucose consumption rate (2.5 to 3.2 x 10^{-17} mol/cell-s), but slightly increased the specific lactate production rate from -3.0 x 10^{-19} mol/cell-s to 10.2 x 10^{-19}

iv

mol/cell-s. Oxidative stress did not contribute to CO_2 inhibition in uninfected Sf-9 cells as no significant increase in the levels of lipid hydroperoxide and protein carbonyl concentrations was discovered at elevated CO_2 concentration. The experimental method used to establish the calibration curve for determining pH_i was well documented. Further investigations are required to determine the role of pH_i in CO_2 inhibition.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure

LIST OF NOMENCLATURE

AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
APOX	Ascorbate peroxidase
BEVS	Baculovirus Expression Vector System
BHK	Baby hamster kidney cells
C*-C	Concentration difference across the gas-liquid interface
$[CO_2]^*$	CO_2 aqueous concentration in equilibrium with the gas phase
CAT	Catalase
CHO	Chinese hamster ovary cells
CuZnSOD	Copper zinc superoxide dismutase
D_i	Impeller diameter
DO	Dissolved oxygen
FBS	Fetal bovine serum
H	Henry's law constant
MnSOD	Manganese superoxide dismutate
N	Agitation speed
N_p	Power number
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
P	Power
P_{CO_2}	Partial pressure of CO_2
PDT	Population doubling time
Re	Reynolds number

ROS	Reactive oxygen species
RQ	Respiratory Quotient
Sf-9	*Spodoptera frugiperda* cell line
Sf-21	*Spodoptera frugiperda* IPLB-Sf21-AE cell line
SOD	Superoxide dismutase
TGF-β	Transforming growth factor beta
T_L	Temperature
Tn-5	*Trichoplusia ni* BTI-Tn-5B1-4 cell line
V	Volume
$k_L a$	Mass transfer coefficient
pH_e	Extracellular pH
pH_i	Intracellular pH
q_{AB}	Specific antibody production rate
q_{amm}	Specific ammonia production rate
q_{gln}	Specific glutamine consumption rate
q_{gluc}	Specific glucose consumption rate
q_{lac}	Specific lactate production rate
rwv	Rotating wall vessel
vvm	Volume of gas flow per volume of liquid per minute
β-gal	β-galactisodase
ρ	Density
μ	Viscosity

CHAPTER – 1

INTRODUCTION

1.1 Insect Cell Lines and Baculovirus

Insect cell lines are widely used to produce eco-friendly biopesticides for controlling agricultural pests and also for the production of complex recombinant proteins using the Baculovirus Expression Vector System (BEVS) (Schlaeger, 1996). The BEVS has many advantages over mammalian expression systems due to its ease of use, safety and its ability for high expression levels. Although over 500 baculoviruses have been identified, the expression of recombinant proteins in insect cells is primarily limited to the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (O'Reilly *et al.,* 1992), which was originally isolated from the larvae of *Autographa californica,* i.e., the alfalfa looper. The word *baculo* refers to the rod-shaped capsids of the virus particles that contain double stranded DNA.

The selection of host insect cell lines for recombinant protein expression depends on 1) growth rate, 2) ability to support virus replication and plaque formation, 3) ability to grow in suspension for ease of cell production and scale-up and 4) ability to support recombinant protein synthesis, including post-translational modification and secretion. *Spodoptera frugiperda* IPLB-Sf21-AE (Sf-21), *Spodoptera frugiperda* (Sf-9) and *Trichoplusia ni* BTI-Tn-5B1-4 (Tn-5) insect cell lines are susceptible to AcMNPV infection and are the most widely used with the BEVS for the production of recombinant proteins (Wang *et al.,* 2001). Sf-21 cells were originally isolated from the pupal ovarian tissue of *Spodoptera frugiperda*, commonly known as the fall armyworm (Vaughn *et al.,* 1977). The Sf-9 cell line is a clonal isolate of Sf-21 cells. The Tn-5 cell

line was isolated from the eggs of the cabbage looper, *Trichoplusia ni* (Granados *et al.*, 1994). Tn-5 cell lines are sold by invitrogen and are commercially known as High Five cells.

Insect cells do not have the ability to carry out the complex N-glycan reactions due to the lack of sufficient levels of the required enzymes. Instead of sialylated complex N-glycan forms produced in mammalian cells that contain two or more outer branches containing N-acetyl glucosamine (GlcNAc), galactose (Gal), and sialic acid attached to the core structure, insect cells produce high mannose or truncated structures, containing two to six mannose (Man) residues linked to the core as shown in Figure 1 (Ikonomou *et al.*, 2003; Chang *et al.*, 2003). Incomplete glycosylation results in improper protein folding, protein stability and cell-cell adhesion. This problem has been addressed by genetically transforming insect cells with mammalian genes that can perform complete glycosylation (Hollister *et al.*, 2001; Hollister *et. al.*, 1998; Chang *et al.*, 2003). Recently, Invitrogen introduced Mimic™ Sf-9 insect cells that are capable of performing complex glycosylation in a manner similar to mammalian cells. Mimic™ cell lines are derived from Sf-9 insect cells and were engineered to produce recombinant proteins with terminally sialylated N-glycans like those found in mammalian systems.

1.2 Cell Metabolism

The growing demands for large-scale insect cell culture for producing recombinant proteins and biopesticides has led to a need for media that supports industrial scale cell culture. Information on insect cell metabolism is necessary to optimize medium formulation. Glucose is considered to be the critical source of carbohydrates in insect cell growth (Ikonomou *et al.*, 2003; Rhiel *et al.*, 1997). The

specific glucose consumption in Sf-9 and Tn-5 cell lines range from 1.7 to 2.4 x 10^{-17} mol/cell-s and 2.8 to 7.4 x 10^{-17} mol/cell-s, respectively. The consumption of sucrose by insect cells is minimal whereas, maltose was completely used during the exponential growth phase. The accumulation of lactate in Tn-5 cell culture is higher than in Sf-9 cell culture. Sf-9 cells produce lactate in stressed environments, e.g., in oxygen depleted cultures (Rhiel *et al.*, 1997).

Insect cells use amino acids for the production of energy and biosynthesis, but cannot synthesize most of the amino acids by themselves. Glutamine is consumed in major quantities by both Sf-9 and Tn-5 cells, while asparagine is consumed in major quantities only by Tn-5 cells. Alanine accumulates in both Sf-9 and Tn-5 cell cultures. However, accumulation of ammonia occurs only in Tn-5 cell cultures. Figure 2 shows the components of cell culture medium that are consumed and produced by the Sf-9 and Tn-5 cells during growth propagation (Rhiel *et al.*, 1997).
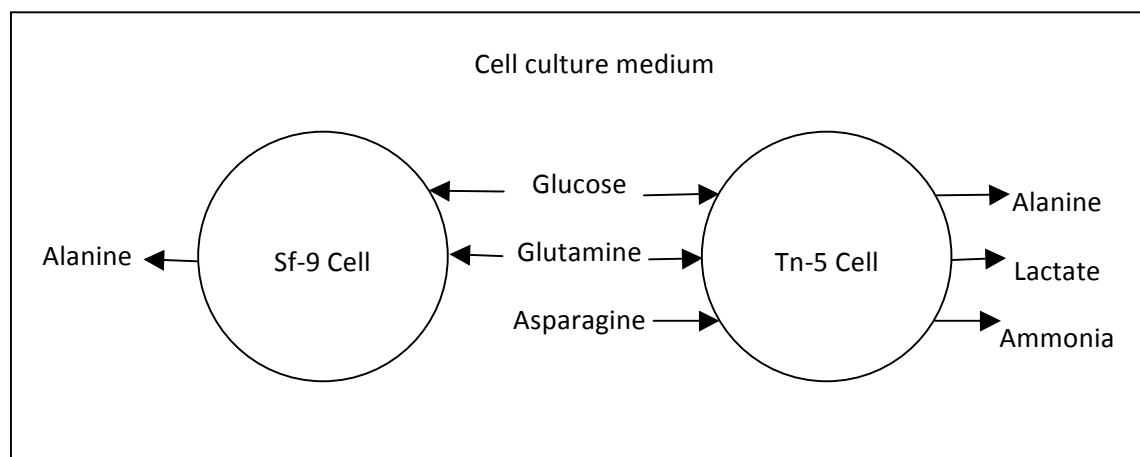
Figure 1: Major metabolic components consumed and produced by Sf-9 and Tn-5 cells during normal growth condition1s

Cellular metabolism for given process conditions is often measured by the Respiratory Quotient (RQ), which is defined as the molar ratio of CO_2 produced to O_2

utilized. The oxygen consumption in Sf-9 and Tn-5 cell lines range between 2 to 5 x 10^{-17} mol/cell-s and 5 to 16 x 10^{-17} mol/cell-s, respectively (Rhiel *et al.*, 1997). The accumulation of CO_2 is more rapid in Tn-5 cell culture than Sf-9 cell culture due to the higher rate of metabolism (Michell-Logean *et al.,* 1997). The RQ of Sf-9 cell line was estimated as ~1, with a CO_2 production rate of 6.1 to 8.6 x 10^{-17} mol/cell-s and the oxygen consumption rate of 5.5 to 8.06 x 10^{-17} mol/cell-s (Kamen *et al.,* 1991).

1.3 Medium Formulation

The first significant contribution to medium development for insect cell culture was made by Wyatt (Wyatt, 1956) and was based on the composition of silkworm hemolymph. This medium had high concentrations of organic salts, inorganic salts, sugars and amino acids and was supplemented with hemolymph. The medium developed by Wyatt was improved with the addition of vitamins (Grace, 1962) and with the addition of protein hydrolysates (yeast extract, casein, lactalbumin, tryptose, etc.) (Mitsuhashi and Maramorosch, 1964). This medium was further modified (designated as TNM-FH medium) by replacing the medium hemolymph with heat-inactivated Fetal Bovine Serum (FBS), removing the organic acids and adding glucose as the main carbohydrate source (Hink, 1970). Later, a more complex IPL-41 medium was developed based on Goodwin's original IPL formulation and was supplemented with a yeastolate-lipid mixture (Weiss *et al.,* 1981). In addition to the above components, Pluronic F-68 is added to the cell culture medium to prevent cell damage in sparged bioreactors (Maiorella *et al.,* 1988; Murhammer and Goochee, 1988). The final pH and osmolality of IPL-41 was adjusted to 6.2 and 365-370 mOsm, respectively. For several years, Grace's medium, TNM-FH medium and IPL-41 medium, supplemented with 5 – 10 % FBS, were

used to carry out insect cell cultures. The composition of Grace's medium and IPL-41 medium was given by Schlaeger (Schlaeger, 1996).

The addition of serum to the cell culture medium promotes cell growth and prevents general stress induced by air bubbles, shear forces and free radicals (Schlaeger, 1996; Donaldson *et al.,* 1998). However, it has many disadvantages, including scale-up problems as it is expensive and in limited supply. The removal of serum from insect cell culture medium reduces the cost, lot-to-lot variability in serum content, potential risk of contamination from mycoplasma and the difficulty in the downstream processing of recombinant proteins. Thus, serum-free media, which provide an economical method of using insect cell culture for the large-scale production of recombinant proteins, have been formulated.

The essential nutrients of most common insect cell culture media are glucose, glutamine, lactalbumin, yeast extract, tryptose, lipids and Pluronic F-68 (Donaldson *et al.,* 1998). Protein hydrolysates have been a substitute for FBS, providing all essential ingredients vital for cell growth. Most of the serum-free media are developed based on IPL-41. Schlaeger developed a low cost serum free medium SF-1 containing Primatone RL which is an enzymatic meat digest (Schlaeger, 1996). Ikonomou *et al.* (2001) developed a serum-free medium, called YPR, which contains yeastolate extract and Primatone RL in major concentrations. YPR serum-free medium prolonged the stationary phase in Sf-9 and Tn-5 cells and also promoted recombinant protein production. Similarly, Donaldson and Shuler used a Hy-Soy protein hydrolysate to develop ISYL serum-free medium for the growth of Tn-5 cells (Donaldson *et al.,* 1998). Presently, several serum-free media, e.g., Express Five SFM, Insect-XPRESS, EX-Cell

400, and Ex-Cell 405 are commercially available. Ex-Cell 401 and Sf-900 II are the most commonly used serum-free media and are supplied by Invitrogen (Schlaeger, 1996). The color of the insect cell medium is usually clear to yellow due to the supplementation with protein hydrolysates. Generally, insect cells are cultured at 27°C to attain optimal cell growth and productivity.

1.4 Large-scale Cell Culture

Practical application of the BEVS technology requires cultivating insect cells on large scale. The large-scale cultivation of insect cells involves many factors, e.g., insect cell lines that can grow in suspension, formulation of low-cost serum-free or protein-free media, and building large bioreactors that can provide a suitable environment for insect cell growth (Agathos, 1996).

1.4.1 Bioreactor Operation

There are three basic modes of bioreactor operation – batch, fed-batch and perfusion (Asenjo, 1995). In a traditional batch operation, the bioreactor is initially seeded with cells and the products are withdrawn only after the run is complete (nothing is added or removed in the interim). In a fed-batch process nutrients are added intermittently to the culture (Ikonomou, 2003) and in a perfusion bioreactor the medium is replenished continuously with the cells retained within the bioreactor. The major advantage of perfusion bioreactors is that they continuously supply nutrients and remove toxic byproducts, thereby allowing the achievement of high cell densities that can lead to high protein production levels. However, operating a perfusion bioreactor is expensive due to the continuous addition and removal of medium, operating equipment like pumps and membranes to supply medium to the bioreactor and also the extra cost to treat the

waste medium (Shuler and Kargi, 2006). Though perfusion is a potential technology for large-scale cell culture, very few biopharmaceutical companies are using this technology because of the concerns over low good manufacturing production, as the usage of additional operating equipment increases the chances of contamination (Marks, 2003). Hence, most industrial processes opt for batch or fed-batch bioreactor operating techniques (Asenjo, 1995).

1.4.2 Bioreactor Types and Scale-up

Different types of bioreactors, e.g., rotating wall vessels, wave bioreactors, airlift bioreactors, bubble column bioreactors and agitated bioreactors, are available for insect cell culture (Agathos, 1996). A rotating wall vessel (rwv) consists of a cylindrical vessel rotating around a horizontal axis. This provides a low shear environment in which cells are maintained in suspension. Both batch and perfusion rwv's are available. Wave bioreactors consist of a rocking platform that generates waves. These waves keep cells in suspension and provide uniform distribution of oxygen and nutrients. In an airlift bioreactor the cells are kept afloat by passing air from the bottom of the bioreactor (Shuler and Kargi, 2006). Stirred-tank bioreactors consist of an impeller to keep the cells suspended and to provide uniform concentration of oxygen throughout the bioreactor.

Bioreactors are scaled-up based on the information obtained from small-scale systems. The principal concerns involved in scaling-up a bioreactor are to maintain uniform nutrient concentrations throughout the bioreactor, to keep cells in suspension, and to provide sufficient oxygen supply while not damaging the cells.

1. The most common criteria for bioreactor scale-up is constant power per volume of liquid, the power imparted by the impeller, is defined by

$$P/V = N_p \, N^3 \, D_i^5 \, \rho \, / \, V, \qquad\qquad (1.1)$$

where N_p = impeller power number, N = agitation speed, D_i = impeller diameter, ρ = medium density and V = medium volume (Marks, 2003; Shuler and Kargi, 2006). The power input depends on impeller speed, impeller type, impeller diameter, presence of baffles and fluid properties. Therefore, the change of scale changes the physical environment inside bioreactor and different scale-up parameters give different results.

2. Maintaining a constant mass transfer coefficient (k_La) is another approach to bioreactor scale-up to ensure the constant oxygen supply to the cells.

3. Other parameters that can be used to scale-up a bioreactor include

    a. Constant impeller tip speed,

    b. Constant height-to-diameter ratio, and

    c. Constant Reynolds number ($Re = \rho D^2 N/\mu$) (Ju *et al.,* 1992).

1.5 Mass Transfer in Stirred-tank Bioreactors

Oxygen is a key nutrient, in addition to other nutrients like glucose, for the growth of all aerobic organisms. The consumption of oxygen is generally represented by OUR, Oxygen Uptake Rate, and is usually reported in moles of oxygen consumed per cell per second. The OUR values vary widely based on the type of medium used, presence of serum in the medium and also baculovirus infection. The Oxygen Transfer Rate (OTR) within the bioreactor should meet with the demands of the OUR. The OTR is directly proportional to the mass transfer coefficient, k_La and the driving concentration difference (C^*-C) across the gas-liquid interfacial area ($OTR = k_La \, (C^*-C)$). In scaled-up

bioreactors, the required oxygen demand can be met by enriching the sparge gas with oxygen, by increasing the agitation rate, or by increasing the gas flow rate.

1.5.1 Agitation and Hydrodynamic Shear

Most biopharmaceutical manufacturing companies use stirred-tank bioreactors. The impeller in a stirred tank bioreactor should be able to provide sufficient agitation to keep the cells suspended, to distribute nutrients uniformly and also to provide sufficient oxygen supply (Shuler and Kargi, 2006). The two conventional impellers used in stirred-tank bioreactors are radial flow impellers (e.g., a flat-blade impeller) and axial flow impellers (e.g., a marine impeller). Radial flow impellers result in fluid flow in a radial direction while axial flow impellers result in vertical fluid flow. Radial flow impellers, e.g., Rushton impellers, are predominantly used for microbial cell culture and low-shear marine impellers are preferred for shear sensitive animal cell lines. Axial flow impellers increase the mass transfer by breaking up the bubbles and increasing their residence time (Marks, 2003). If required, a second or even a third impeller can be added to provide sufficient agitation and also to avoid eddy formation. Sufficient spacing between the impellers and also between the impeller and liquid surface is required to avoid vortex formation. Vortex formation can also be prevented by using a scoping impeller; which is a specialized type of marine impeller.

Animal cells, unlike microbial cells, lack a protective cell wall and are therefore more susceptible to physical damage. The two primary mechanisms that can cause physical damage to insect cell lines are high hydrodynamic shear stress and bubble damage. Studies have shown that the cell damage caused by hydrodynamic forces is often negligible when compared to the cell damage caused by bursting bubbles (Marks,

2003). The agitation rate for animal cell culture should be selected to provide proper mixing with minimal hydrodynamic shear effects on cell lines. Investigations have demonstrated that moderate agitation has negligible effect on Sf-9 cell growth (Murhammer *et al.,* 1990; Murhammer *et al.,* 1988). Higher agitation rates increase the mass transfer area and promote good oxygen supply by preventing bubble coalescence, decreasing the thickness of the gas-liquid interface film, and by increasing the bubble break-up and bubble hold-up. However, higher agitation rates induce vortex formation, cavitation and foam accumulation that can result in rapid cell death. The cell damage caused by vortex formation and cavitation can be prevented with the addition of 0.2% Pluronic F-68 to the cell culture medium.

1.5.2 Aeration and Bubble Damage

Oxygen supply to bioreactor can be done in three ways: surface aeration, membrane aeration or sparged aeration. In surface aeration air and/or oxygen is supplied through the bioreactor headspace. Surface aeration does not cause any bubble damage to cells, but is only effective in supplying sufficient oxygen in small-scale systems, i.e., those with a large surface area to volume ratio. In membrane aeration air and/or oxygen is introduced through permeable silicone tubes. Membrane aeration is also limited to small-scale systems (Marks, 2003). In sparged aeration, air and/or oxygen is directly released into the cell culture medium via bubbling.

Sparging is the most effective way of supplying oxygen to large-scale bioreactors. Several different types of spargers are available, e.g., point, frit and ring spargers. The flow rate of a gaseous mixture through the sparger is usually measured in volume of gas flow per volume of liquid per minute (vvm). Smaller bubbles provide better oxygen

transfer, but are more likely to damage the cells. On the other hand, oxygen transfer from larger bubbles is less efficient, but is less likely to damage the cells. Nevertheless, several parameters influence the cell damage, e.g., the magnitude of energy dissipated during bubble break-up, bubble size, bubble residence time and the probability of bubble breakup. Furthermore, foaming problems are more likely with smaller bubbles. Foaming can be reduced with the addition of anti-foaming agent and bubble damage can be reduced with the addition of surfactants, e.g., Pluronic F-68. In addition to the foaming and cell damage issues, bubble size also influences the accumulation of gaseous metabolic byproducts like CO_2, which are mainly removed by stripping from the gas-liquid mass transfer area present around the bubbles. In large scale bioreactors (especially tall and small diameter bioreactors) smaller bubbles often dissolve in the cell culture medium, without stripping CO_2 before reaching the surface (Gray *et al.,* 1996). Hence, the size of the bubbles produced by a sparger should be optimized such that it provides sufficient oxygen supply, does not harm the cells, and removes gaseous metabolic byproducts.

1.5.3 Major Scale-up Obstacle and CO_2 Accumulation

The greater constraints existing on agitation and sparge flow rates in animal cell cultures increase the difficulty of providing sufficient oxygen without damaging cells in large scale and high cell density cultures. To overcome this problem reduced sparge rates are used with pure oxygen to provide enough oxygen supply in large-scale animal cell cultures. Reduced sparge rates reduces the gas flow that can prevent CO_2 accumulation within the bioreactor.

CO$_2$ accumulation increases with the increase in bioreactor volume and also with the increase in cell densities, thereby increasing the inhibition of cell growth and productivity in both insect and mammalian cell bioreactors (Gray *et al.,* 1996; Garnier *et al.,* 1996; Kimura and Miller, 1996). In mammalian cell culture, higher CO$_2$ concentration levels decreased cell density, viability and specific productivity in Chinese Hamster Ovary (CHO) cells (Gray *et al.,* 1996). In baculovirus infected Sf-9 insect cells, elevated CO$_2$ concentration levels decreased the production of β-galactisodase (β-gal) and Transforming Growth Factor beta (TGF-β) recombinant proteins (Garnier *et al.,* 1996).

The accumulation of CO$_2$ and its dissociation into HCO$_3^-$ and H$^+$ ions, in cell culture medium and in the cell cytoplasm, may induce oxidative stress, decrease intracellular pH (pH$_i$) and alter medium osmolality. The role of CO$_2$ accumulation in oxidative stress is primarily due to the formation of carbonate anion (CO$_3^{\bullet-}$) and peroxymonocarbonate anion (HCO$_4^-$) (Goss *et al.,* 1999; Vesela and Wilhelm, 2002). The formation of CO$_3^{\bullet-}$ and HCO$_4^-$ occurs mainly in the presence of strong oxidizing agents like H$_2$O$_2$. The viral infection of cells produces significant levels of H$_2$O$_2$ and hence would be expected to lead to the production of CO$_3^{\bullet-}$ and HCO$_4^-$ in the presence of HCO$_3^-$. CO$_3^{\bullet-}$ and HCO$_4^-$ are strong oxidizing components and can damage proteins and lipids, thereby altering cell metabolism. The formation of CO$_3^{\bullet-}$ and HCO$_4^-$ occurs due to the following processes:

1. Production of carbonate anion (CO$_3^{\bullet-}$)

    a. H$_2$O$_2$ can be converted to hydroxyl radical (OH$^\bullet$) by the following mechanism:

$$\text{i.} \quad SOD - Cu^{2+} + H_2O_2 \ \leftrightarrow \ SOD - Cu^{1+} + O_2^{\bullet -} + 2H^+ \tag{1.2}$$

$$\text{ii.} \quad SOD - Cu^{1+} + H_2O_2 \ \leftrightarrow \ SOD - Cu^{2+} - OH^\bullet + OH^- \tag{1.3}$$
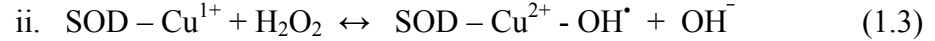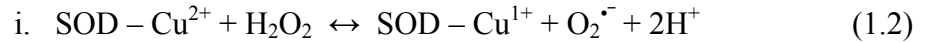
    b. The resulting $OH^\bullet$ oxidizes HCO_3^- to produce $CO_3^{\bullet -}$:

$$\text{i.} \quad SOD - Cu^{2+} - OH^\bullet + HCO_3^- \ \leftrightarrow \ SOD - Cu^{2+} + H_2O + CO_3^{\bullet -} \tag{1.4}$$
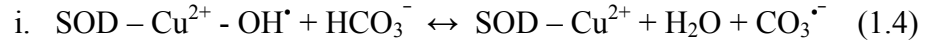
2. Production of peroxymonocarbonate anion (HCO_4^-)

    a. H_2O_2 Reacts with HCO_3^- to produce HCO_4^-

$$\text{i.} \quad H_2O_2 + HCO_3^- \ \leftrightarrow \ H_2O + HCO_4^- \tag{1.5}$$
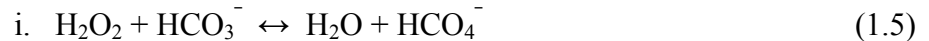
The increase in CO_2 concentration leads to intracellular acidification in mammalian cells due to the accumulation of higher levels of H^+ ions in the cell cytoplasm (deZengotita *et al.*, 2002; Taticek *et al.*, 1998 and Adler *et al.*, 1965). The decreased pH_i (increase in H^+ ions) has detrimental effects on cellular metabolism by interfering with the enzyme activity. The contribution of CO_2 accumulation to medium osmolality is largely due to the maintenance of cell culture at constant pH, i.e., through the addition of base. In CHO cell cultures the combination of CO_2 accumulation and elevated osmolality resulted in decreased CHO cell growth, metabolism, and productivity (Kimura *et al.,* 1996; deZengotita *et al.,* 1998). Insect cells are relatively insensitive to changes in osmolality from 250 to 400 mOsm/kg (Gardiner *et al.,* 1976). Hence, changes in osmolality caused by CO_2 accumulation are expected to have minimal/no effect on insect cell cultures.

1.6 Objective and Hypothesis:

This research investigated the effect of specific CO_2 concentrations on insect cell growth and metabolism. A well-controlled bioreactor maintained at a constant CO_2 concentration was used to determine the inhibitory effect of CO_2 on uninfected Sf-9 cell

growth and metabolism. Also, the mechanism of the inhibitory effect of CO_2 was studied by evaluating the role of oxidative stress. Furthermore, experiments leading to the determination of the effect of elevated CO_2 on pH_i in insect cells were conducted.

The hypothesis of this research work is that the CO_2 accumulation inhibits cell growth in uninfected insect cell culture and that this results from intracellular acidification and not from increased oxidative stress.

1.7 Overview:

This thesis is focused on the investigation of the effect of elevated CO_2 concentration on uninfected insect cell growth and metabolism and the role of oxidative stress and pH_i in CO_2 inhibition. Chapter 2 explains the previous studies conducted on the CO_2 inhibition in insect and mammalian cells and several different techniques suggested to overcome CO_2 inhibitory affect. Chapter 3 discusses the current research work conducted on the effect and mechanism of CO_2 inhibition in uninfected insect cells including the materials and methods involved in carrying out the experiments, the experimental results and the discussions. Finally, chapter 4 contains conclusions and recommendations for future work.

CHAPTER – 2

LITERATURE REVIEW

Previous studies have demonstrated that CO_2 accumulation inhibits cell growth and recombinant protein production in both insect and mammalian cell bioreactors. The current chapter provides a summary of previous investigations conducted on the effect of CO_2 accumulation on growth and productivity in different cell lines, role of osmolality, oxidative stress and intracellular pH in CO_2 inhibition, and also approaches that can be used to reduce CO_2 accumulation, and/or reduce the adverse effects thereof.

2.1 Sources of CO_2

The physiological concentration of CO_2 that is required for the normal growth of cells is 30-50 mm Hg (i.e., an aqueous phase concentration in equilibrium with a gas phase with a CO_2 partial pressure of 30-50 mm Hg). The accumulation of higher CO_2 concentrations in bioreactors is primarily due to the addition of CO_2 from biotic sources, e.g., cellular respiration, and the addition of CO_2 from abiotic sources, e.g., addition of bicarbonate as a buffer to the cell culture medium and addition of CO_2 or $NaHCO_3$ to control the pH of the cell culture medium (Goudar *et al.,* 2006).

2.2 Role of Osmolality in CO_2 Inhibition

As CO_2 accumulates, it can easily transfer between the gas-liquid interface and through the cell membrane (Figure 3) and dissociate into bicarbonate (HCO_3^-) and hydronium ions (H^+) in aqueous solutions. The HCO_3^- and the base (e.g., KOH) added to control the pH of cell culture medium contribute to increased medium osmolality. Increased osmolality alters membrane exchanger activity to maintain cell volume, which can lead to increased intracellular solute concentrations. These increased intracellular

solute (e.g., Na^+ or Cl^-) concentrations can then adversely affect intracellular enzyme activity. Hence, increase in CO_2 accumulation can increase the medium osmolality resulting in distorted cell functioning.
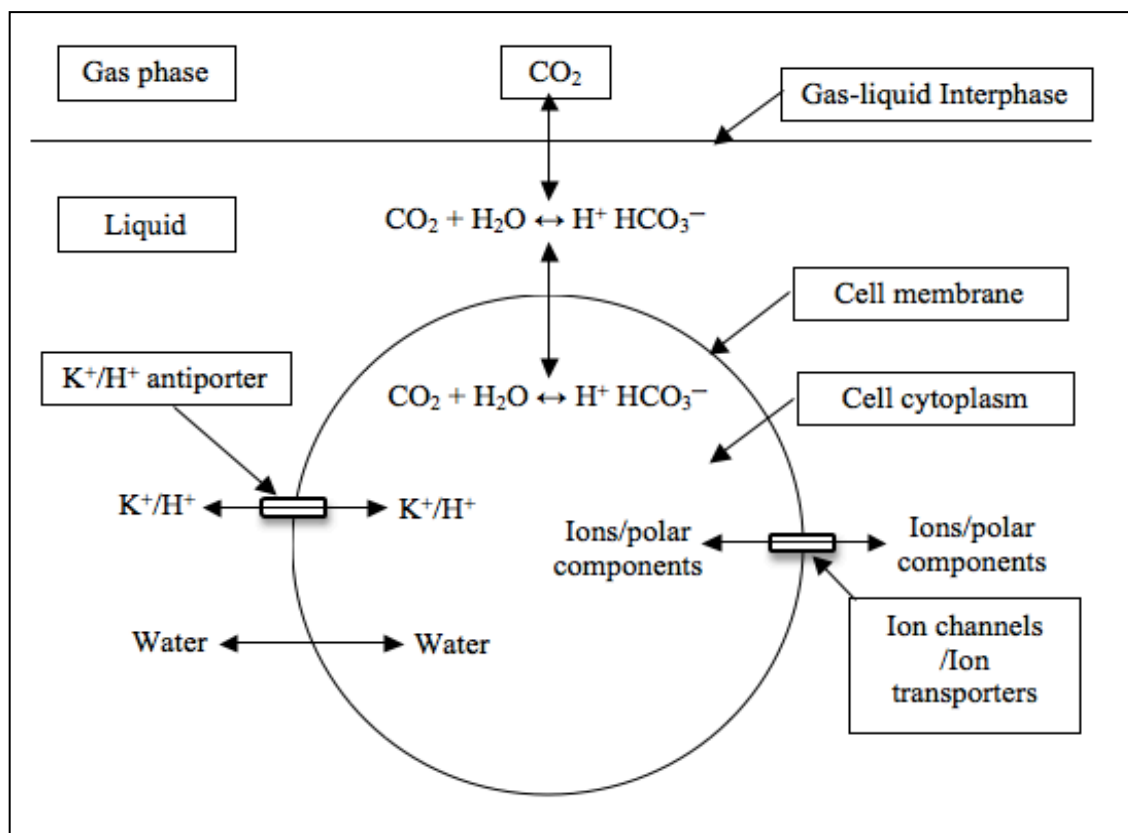
Figure 2: Schematic of CO_2 and ion transfer between gas-liquid interface and cell membrane

2.2.1 Effect of CO_2 and/or Osmolality on Mammalian Cells

Studies conducted in a 10 L CHO cell perfusion bioreactor demonstrated that the productivity was maximized when CO_2 concentrations were maintained in the range of 30-70 mm Hg and decreased cell growth and productivity was observed at a CO_2 concentration of 105 mm Hg (Gray *et al.,* 1996). Increasing the CO_2 concentration from 38 to 145 mm Hg reduced the specific productivity of CHO cells by 50% and resulted in a decrease in the maximum viable cell density from 8.9×10^6 to 5.9×10^6 cells/mL.

Experiments conducted in an incubator controlled at a CO_2 concentration of 0 mm Hg resulted in no cell growth, whereas a CO_2 concentration of 30 mm Hg resulted in normal CHO cell growth. These experiments demonstrated that the presence of lower CO_2 concentrations (around 30 mm Hg) enhances cell growth but the presence of higher CO_2 concentrations (above 100 mm Hg) reduces cell growth and productivity. In addition to this, investigations with CHO cells demonstrated that increased CO_2 concentration decreases cell growth in a dose dependent manner (Drapeau *et al.,* 1990; Kimura and Miller, 1996; Aunins and Henzler, 1993). An increase in CO_2 concentration from 53 to 167 mm Hg decreased the CHO cell growth by 41% (Drapeau *et al.,* 1990), whereas an increase in CO_2 from 36 to 250 mm Hg (at a controlled osmolality of 310 mOsm/kg) decreased the CHO cell growth by 30% (Kimura and Miller, 1996).

2.2.1.1 Cell Growth

Several investigations have demonstrated that a combination of elevated CO_2 concentration and osmolality resulted in reduced cell growth (Kimura and Miller, 1996; deZengotita *et al.,* 1998; Zhu *et al.,* 2005), whereas the individual effect of elevated CO_2 concentration (with lower osmolality levels) and elevated osmolality (with lower CO_2 concentration) had minimal effect on cell growth below a certain threshold value (Kimura and Miller, 1996; Zhu *et al.,* 2005). The experiments conducted on CHO cells at elevated CO_2 concentrations of 140 and 195 mm Hg (at an osmolality of 310 mOsm/kg) resulted in very little or no change in growth rates when compared to the control at a CO_2 concentration of 36 mm Hg (at an osmolality of 310 mOsm/kg). However, the growth rate decreased by 30% with an increase in CO_2 concentration from 36 to 250 mm Hg (at an osmolality of 310 mOsm/kg) (Kimura and Miller, 1996). Similarly, the experiments

conducted at elevated osmolality (342, 357, and 376 mOsm/kg) and lower CO_2 concentration (36 mm Hg) had minimal effect on cell growth (Kimura and Miller, 1996). However, increasing the osmolality from 316 to 445 mOsm/kg (at CO_2 concentrations of 30-70 mm Hg) reduced the growth rate by 60% (Zhu *et al.,* 2005) (Table 1).

The combination of elevated osmolality and elevated CO_2 concentration had a detrimental effect on cell growth (Kimura and Miller, 1996; deZengotita *et al.,* 1998; Zhu *et al.,* 2005). The experiments conducted on CHO cells in T-150 flasks at 250 mm Hg and 376 mOsm/kg resulted in a 45% decrease in the growth rate compared to the control at 36 mm Hg and 310 mOsm/kg (Kimura and Miller, 1996). Experiments conducted in a 3 L bioreactor maintained at 140 mm Hg and 370 mOsm/kg resulted in a decreased growth rate of 72% when compared to the control maintained at 36 mm Hg and 310 mOsm/kg (deZengotita *et al.,* 1998). Furthermore, experiments conducted in a 5 L bioreactor resulted in a 64% decrease of CHO cell growth rate at 168 mm Hg and 375 mOsm/kg when compared to the control at 90 mm Hg and 325 mOsm/kg (Zhu *et al.,* 2005).

Table 1: Effect of different levels of CO_2 concentration and osmolality on CHO cell growth rates

Experimental values CO_2 concentration (mm Hg) / osmolality (mOsm/kg)	Control values CO_2 concentration (mm Hg) / osmolality (mOsm/kg)	Effect on growth rate	Reference
Elevated CO_2 concentration and lower osmolality levels			
140 mm Hg / 310 mOsm/kg	36 mm Hg / 310 mOsm/kg	negligible change	Kimura and Miller, 1996
195 mm Hg / 310 mOsm/kg		negligible change	
250 mm Hg / 310 mOsm/kg		30% decrease	
Lower CO_2 concentration and elevated osmolality levels			
36 mm Hg / 342 mOsm/kg	36 mm Hg / 310 mOsm/kg	negligible change	Kimura and Miller, 1996
36 mm Hg / 357 mOsm/kg		negligible change	
36 mm Hg / 376 mOsm/kg		negligible change	
36-70 mm Hg / 445 mOsm/kg	36-70 mm Hg / 316 mOsm/kg	60% decrease	Zhu et al., 2005
Elevated CO_2 concentration and elevated osmolality levels			
140 mm Hg / 337 mOsm/kg	36 mm Hg / 310 mOsm/kg	22% decrease	deZengotita et al., 1998
140 mm Hg / 370 mOsm/kg		35% decrease	
195 mm Hg / 361 mOsm/kg		45% decrease	
195 mm Hg / 415 mOsm/kg		63% decrease	
250 mm Hg / 376 mOsm/kg	36 mm Hg / 310 mOsm/kg	45% decrease	Kimura and Miller, 1996
168 mm Hg / 375 mOsm/kg	90 mm Hg / 325 mOsm/kg	35% decrease	Zhu et al., 2005

2.2.1.2 Cell Death and Apoptosis

Various contradictory propositions were made regarding the effect of CO_2 and osmolality on cell viability. Experiments conducted in a 10 L bioreactor using CHO cells demonstrated that increasing the CO_2 concentration from 35 to 148 mm Hg (osmolality of 310 to 340 mOsm/kg) decreased the cell viability from 79% to 58% (Gray *et al.,* 1996), whereas experiments conducted in a 2 L bioreactor with CHO cells demonstrated that the increase in CO_2 concentration (60, 100, and 150 mm Hg) at constant osmolality (350 − 370 mOsm/kg) had a negligible effect on CHO cell viability (Zhu *et al.,* 2005). Similarly, investigations conducted with CHO cells at 36 mm Hg CO_2 demonstrated that increased osmolality (357 and 376 mOsm/kg) increased the specific death rate by 1.3 and 1.6 times that of the control (310 mOsm/kg) (deZengotita *et al.,* 1998), whereas cell viability was not affected by increased osmolality (310, 340, 370, 400, 420, and 450 mOsm/kg) at CO_2 concentrations of 30 to 70 mm Hg. Furthermore, specific death rates increased by 2.2, 6.2 and 64 times at 140 mm Hg/370 mOsm/kg, 195 mm Hg/415 mOsm/kg and 250 mm Hg/469 mOsm/kg, respectively, when compared to the control at 36 mm Hg/310 mOsm/kg.

Some investigations suggested that hybridoma cell death under elevated CO_2 concentration and osmolality mainly occurred due to apoptosis (deZengotita *et al.,* 2002). Apoptosis increased with increasing osmolality in a dose dependent manner. Also, the effect of elevated CO_2 concentration on apoptotic cell death was minimal at a constant osmolality. Furthermore, increasing CO_2 concentration along with osmolality did not increase apoptosis beyond that obtained by increasing osmolality alone.

2.2.1.3 Cell Metabolism

In CHO cells elevated CO_2 concentration (at a constant osmolality) decreased the specific glucose (q_{gluc}) and glutamine (q_{gln}) consumption rates and lactate (q_{lac}) production rate in a dose dependent manner. However, elevated CO_2 concentration did not affect the specific ammonia production rate (q_{amm}) (deZengotita *et al.,* 2002). Increased osmolality (at constant CO_2 concentration) was shown to increase q_{gluc}, q_{gln}, q_{lac} and q_{amm} (Zhu *et al.,* 2005). However, other investigations showed that increased osmolality had negligible effect on q_{gluc} and q_{gln} (deZengotita *et al.,* 1998; deZengotita *et al.,* 2002). This apparent discrepancy may be due to different cell culture media used, which have been demonstrated to have different effects on glucose metabolism under elevated osmolality conditions (Ryu and Lee, 1997).

2.2.1.4 Protein Production and Glycosylation

Increased CO_2 concentration decreased the specific productivity of tPA in CHO cells in a dose dependent manner (Kimura *et al.,* 1996). The increase of CO_2 concentration from 36 to 140 mm Hg and 195 mm Hg (osmolality of 310 mOsm/kg) decreased the q_{tPA} by 10% and 33%, respectively. The experiments conducted in a 10 L perfusion reactor for production of an antigen from CHO cells demonstrated that the increase of CO_2 concentration from 35 to 148 mm Hg (no osmolality measure was taken) decreased the specific productivity and the total productivity by 44% and 70%, respectively (Gray *et al.,* 1996). Also, the increase of CO_2 concentration from 60 to 120 mm Hg in a 200 L vessel decreased the volumetric IgG production of recombinant NSO myeloma cells by 20%, but did not affect the per cell IgG productivity (Aunins and Henzler, 1993).

Elevated osmolality induces stress in hybridoma cells and increases the specific antibody production rate (Oyaas et $al.,$ 1994; Reddy an Miller, 1994; deZengotita et $al.,$ 2002; Kim and Lee, 2002; Zhu et $al.,$ 2005). The specific production of IgG_2 increased with increasing osmolality (at a constant CO_2 concentration) in a dose dependant manner (deZengotita et $al.,$ 2002; deZengotita et $al.,$ 1998). Similar results were obtained during the production of B1 protein (Zhu et $al.,$ 2005). Although elevated osmolality increased the specific antibody production, it did not result in increased final product concentration due to reduced cell growth and viability at elevated osmolality (Zhu et $al.,$ 2005).

Inconsistent arguments have been made regarding the combined effect of elevated CO_2 concentration and osmolality. Some investigations demonstrated that the specific antibody production rate (q_{AB}) increased at higher CO_2 concentrations without osmolality compensation (deZengotita et $al.,$ 2002; deZengotita et $al.,$ 1998; Kimura and Miller, 1996). The increase of osmolality from 310 to 370 mOsm/kg (at CO_2 concentration of 250 mm Hg) increased the q_{tPA} by 30% (Kimura and Miller, 1996). Furthermore, specific IgG_{2a} production increased by 20 to 25% at 140 mm Hg and 370 mOsm/kg and 195 mm Hg and 415 mOsm/kg compared to 36 mm Hg and 310 mOsm/kg (deZengotita et $al.,$ 1998), respectively. However, other studies demonstrated that elevated CO_2 concentration (140-160 mm Hg) and elevated osmolality (400-450 mOsm/kg) together had no significant effect on the final product yield (Zhu et $al.,$ 2005).

The effect of elevated CO_2 concentration and osmolality on glycosylation was also investigated (Kimura and Miller, 1997; Zhangi et $al.,$ 1999). Increasing CO_2 concentration from 36 to 250 mm Hg had no effect on total sialic acid content, the expression of high mannose oligosaccharides, or the distribution of surface charges

(Kimura and Miller, 1997). However, the production of sialic acids comprising N-glycolyl-neuraminic acid (Neu5Gc) at 250 mm Hg decreased by 40% compared to the control (36 mm Hg). Furthermore, increasing CO_2 concentration from 38 to 120 mm Hg, decreased the polysialic acid (PSA) content by 40% (Zhangi *et al.,* 1999).

2.2.2 Effect of CO_2 and/or Osmolality on Insect Cells

Investigations conducted on baculovirus infected Sf-9 insect cells demonstrated that the quantity of recombinant protein produced in a 150 L bioreactor was only 30% of that produced in a 70 mL spinner flask (Garnier *et al.,* 1996). Also, the effect of elevated CO_2 concentration on the production of β-gal and TGFβ-RII-ED recombinant proteins from baculovirus infected Sf-9 cells was studied in an incubator controlled at 15% CO_2 in air. The production of β-gal and TGFβ receptor at 15% CO_2 reduced by 61% and 44%, respectively, when compared to the control (incubator with pure air). Although the elevated CO_2 environment decreased the total recombinant protein production, it prolonged cellular viability and extended the expression phase. It was proposed that the elevated CO_2 concentrations altered the cellular metabolism and delayed the infection process, thereby prolonging the cellular viability and recombinant protein production.

Insect cells are generally insensitive to large changes in medium osmolality, e.g., osmolality ranging from 250 – 400 mOsm/kg had minimal effect on *Trichnoplusia ni* Tn-368 cell growth (Gardiner *et al*., 1976). Increasing the medium osmolality to 510 mOsm/kg with the addition of 30 mM NaCl to EX-CELL 405 serum-fee medium had minimal effect on cell growth and a slight inhibitory effect on recombinant protein synthesis in the Tn-5 cell line (Yang *et al*., 1996). Therefore, changes in osmolality due to CO_2 accumulation are expected to have minimal/no effect on insect cell cultures.

2.3 Role of Intracellular pH in CO_2 Inhibition

The activity of several cellular metabolic enzymes, e.g., phosphofructokinase, are pH sensitive. Changes in intracellular pH (pH_i) affects cell metabolism, ion conductivities, protein synthesis and cell cycle (Madshus *et al*., 1988). The intracellular dissociation of CO_2 results in the accumulation of H^+ ions in the cell cytoplasm leading to reduce intracellular pH (pH_i) even when the extracellular pH (pH_e) is controlled. Although Na^+/H^+, Cl^-/HCO_3^- and similar antiporters counteract the intracellular acidification effect in mammalian cells (Madshus *et al*., 1988), the pH_i may not return to its original value (Krapf *et al*., 1988). The presence of K^+/H^+ antiporter plays a key role in the transport of H^+ ions across the cell membrane in Sf-9 insect cells (Vachon *et al*., 1995).

Several investigations conducted on mammalian cells confirmed that increased CO_2 concentration decreases pH_i (deZengotita *et al*., 2002; Taticek *et al*., 1998; Adler *et al*., 1965). Studies conducted on BHK-21 cells maintained at pH_e 6.8 in perfusion culture showed that increase in CO_2 concentration from 35 mm Hg to 270 mm Hg decreased the pH_i from 7.2 to 6.9 (Taticek *et al*., 1998). In contrast, experiments conducted on hybridoma cells in T-flasks demonstrated that increased CO_2 concentration (up to 140 mm Hg) decreased pH_i by 0.1 – 0.2 units and that pH_i did not decrease further with increased CO_2 concentration above 140 mm Hg.

The pH_i of Sf-9 cells grown in Grace's insect cell culture medium (pH 6.5) was reported as 6.3 (Vachon *et al*., 1995). The pH_i of Sf-9 cells is largely dependant on the medium composition especially the extracellular concentration of K^+. However, previous

investigations conducted demonstrated that the pH_i of uninfected Sf-9 cells grown in Sf-900 II medium (pH 6.4) was reported as 6.7.

2.4 Role of Oxidative Stress in CO_2 Inhibition

Reactive Oxygen Species (ROS), e.g., superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($^\bullet OH$), and hydrogen peroxide (H_2O_2), are the normal byproducts of cellular metabolism. The ROS produced by insect cell lines are detoxified by the production of antioxidant enzymes, e.g., superoxide dismutase (MnSOD and CuZnSOD that reduces $O_2^{\bullet-}$ to H_2O_2) and ascorbate peroxidase (APOX that reduces H_2O_2 to H_2O) that are present in both Sf-9 and Tn-5 cells (Wang *et. al.,* 2001). Additionally, Tn-5 cells contain catalase (CAT) for reducing H_2O_2 to H_2O. The imbalance between the production of ROS and the ability to detoxify them creates oxidative stress. Excessive free radicals damage cellular components, e.g., protein and lipids. The AcMNPV infection of Sf-9 and Tn-5 cells resulted in high levels of lipid hydroperoxides and protein carbonyls, which are indicative of oxidative stress (Wang *et. al.,* 2001).

Bicarbonate ions, resulting from CO_2 accumulation, combine with H_2O_2 (produced as a result of the viral infection of insect cells) to form strong oxidizing components, e.g., $CO_3^{\bullet-}$ and HCO_4^-, thus inducing oxidative stress (eq. 1.2 – 1.5). Hence, it was hypothesized that CO_2 accumulation induces oxidative stress only in infected insect cells.

2.5 CO_2 Reduction Strategy

The dissolved CO_2 concentration in high cell density CHO and BHK cell cultures can be significantly reduced by eliminating the CO_2 addition from abiotic sources (Matanguihan *et al.,* 2002; Goudar *et al.,* 2006). The addition of CO_2 or $NaHCO_3$ to

control pH can be replaced with the addition of Na_2CO_3. The addition of bicarbonate buffer can be substituted by histidine or iminodiacetic acid as complexing agents and 3-(N-morpholino)-propanesulfonic acid (MOPS), N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (TES), tris-(hydroxymethyl)-aminoethane (TRIZMA), or N-(2-hydroxyethyl)-piperazine-N-2-ethanesulfonic acid) (PIPES) as buffers, depending on the suitability of composition for cell growth, metabolism and productivity. The replacement of bicarbonate buffer with MOPS-histidine buffer and $NaHCO_3$ (used for pH control) with Na_2CO_3 reduced the dissolved CO_2 concentration from ~230 mm Hg to 68-85 mm Hg and increased the growth and productivity of BHK cells from 68 to 123% and 58 to 90%, respectively (Goudar *et al.,* 2006).

Substantial elimination of CO_2 can be achieved by increasing the bubble size, sparge flow rates, and impeller position (Mostafa and Gu, 2003; Gray *et al.,* 1996). Dissolved CO_2 was efficiently removed from a 500 L CHO cell perfusion bioreactor by using pure oxygen flow through the headspace and through the sparger and by increasing the bubble size from 2 μm to 2-3 mm and sparge flow rates from 0.002 to 0.006 vvm, which provided adequate oxygen supply along with CO_2 removal (Gray *et al.,* 1996). Similarly, in a CHO cell fed-batch culture, CO_2 was removed by macro sparging (Mostafa and Gu, 2003).

Dissolved CO_2 concentration can also be reduced by purging the headspace of the bioreactor with air (Mitchell-Logean and Murhammer, 1997). Headspace purging increased the growth rates and the maximum viable cell densities in Sf-9 and Tn-5 insect cell cultures and reduced the dissolved CO_2 concentrations from 600 to ~75 mm Hg (based on a CO_2 solubility of 25 mm Hg/mM in insect cell culture medium at 27°C)

(Onda *et al.,* 1970). The mean Population Doubling Times (PDT) of Sf-9 and Tn-5 cells decreased from 38 to 22 h and from 46 to 22 h, respectively. The maximum viable cell densities increased from ~5-6 to ~8-10 x 10^6 and from ~3-5 to ~8-10 x 10^6 cells/mL in Sf-9 and Tn-5 cells, respectively. It was also proposed that the decline in growth rates of insect cells in the presence of high dissolved CO_2 concentrations is due to growth inhibition rather than cell death as the cell viability was above 90% at all times.

The inhibitory effect of elevated CO_2 concentration can be reduced by the adaptation of cells to higher CO_2 concentrations (Croughan, 2009). A few cells within a cell population possess the ability to adapt to a wide range of conditions that are fatal to nearly all the original cell population. Similarly, continuous exposure of cells to higher CO_2 concentrations over a period of time (a few months) allows the cells to adapt to grow in higher CO_2 concentrations.

Another approach to overcome the inhibition of high dissolved CO_2 concentrations is to find the mechanism of CO_2 inhibitory effect. pH_i (both in uninfected and in infected cells) and oxidative stress (in infected cells) are predicted to be the possible sources of CO_2 inhibition. If significant levels of pH_i or oxidative stress are found, then further experiments can be conducted to decrease the CO_2 inhibitory effect by increasing the appropriate antiporter activity or by the expression of antioxidant enzymes in cells, respectively, by cell transfection with the corresponding genes.

2.6 Current Research Contribution

Most of the previous reviews focused on the effect of CO_2 inhibition in mammalian cells and very few studies have been conducted in insect cells. One of the shortcomings of the previous investigations was the method in which dissolved CO_2

concentration was measured. Specifically, the CO_2 concentration was either measured offline, e.g., with a blood gas analyzer, or by estimating the CO_2 concentration based on mass transfer equations (i.e., known gas phase concentrations used to estimate liquid phase concentration). These measurement methods are not expected to be as accurate as direct measurement, as in the studies discussed in this thesis. Another shortcoming results from the fact that most of the previous investigations involved controlling the CO_2 concentration in the gas-phase; thus, the effect of a specific CO_2 concentration in the cell culture medium was not determined. Furthermore, many investigations were conducted (especially in CHO cells) on the role of osmolality and CO_2 accumulation on the observed inhibitory effect but none of the previous reviews investigated the role of oxidative stress or intracellular pH in the CO_2 inhibitory effect.

The present research study investigates the effect of specific CO_2 concentrations in insect cell culture by using a well-controlled bioreactor. An autoclavable CO_2 probe was used to accurately measure the dissolved CO_2 concentration within the bioreactor at any given time. The role of oxidative stress in the CO_2 inhibitory effect was investigated by monitoring lipid peroxide and protein carbonyl formation. Also, experiments were conducted to find the effect of elevated CO_2 concentration on pH_i in insect cells.

CHAPTER – 3

MECHANISM OF CO_2 INHIBITION IN INSECT CELL CULTURE

3.1 Introduction

The prominence of insect cell culture has grown rapidly in recent years due to its ability to produce baculovirus biopesticides and recombinant proteins using the Baculovirus Expression Vector System (BEVS). Large-scale production of these products involves high cell density ($\sim 20 \times 10^6$ cells/mL) bioreactors with large working volumes (10,000 L – 100,000 L). One of the critical problems in using large scale and high cell density cultures is the accumulation of CO_2 to inhibitory levels within the bioreactor. Cellular metabolism is the primary source of CO_2 accumulation, whereas the bicarbonate (added as a buffer) addition is a secondary source. The respiratory quotient, i.e., the molar ratio of CO_2 produced to O_2 consumed which is typically ~1 for Sf-9 cells, can be used to estimate the cellular CO_2 production rate if the dissolved O_2 rate is known (Kamen *et al.,* 1991; Aunins and Henzler, 1993).

Insect and mammalian cell lines can tolerate only limited agitation and sparge flow rates due to their shear sensitive nature (Murhammer, *et al.,* 1988). Hence, small bubbles of pure oxygen are generally used in large-scale insect and mammalian cell bioreactors in order to provide sufficient oxygen (Murhammer, *et al.,* 1990; Marks, 2003). These small bubbles often dissolve in the cell culture medium before reaching the surface without stripping CO_2, thereby leaving surface mass transfer as the only means of CO_2 removal. Also, in large-scale bioreactors the surface to volume ratio decreases with increasing bioreactor size, thereby further reducing dissolved CO_2 removal to exacerbate CO_2 accumulation within the bioreactor.

As CO_2 accumulates in the bioreactor, it dissolves in the cell culture medium as well as in the cell cytoplasm (i.e., CO_2 passes freely across cell membranes) and dissociates into HCO_3^- and H^+ ions (Equation 3.1).

$$CO_2 + H_2O \leftrightarrow H^+ + HCO_3^- \qquad (3.1)$$

The solubility of a gas in a solvent is directly proportional to the partial pressure of that gas above the solvent and is given by Henry's law (Equation 3.2).

$$P_{CO2} = H * [CO_2]^* \qquad (3.2)$$

Where $[CO_2]^*$ is the CO_2 concentration in equilibrium with the gas phase, P_{CO2} is the partial pressure of CO_2 and H is the Henry's law constant (Pa/M). The Henry's law constant for CO_2 dissolved in pure water at 27^oC (temperature of insect cell culture) was calculated as 23.2 mm Hg/mM by using the equation given by Sperandio and Paul (1997) (Equation 3.3).

$$H = 101,330 * exp [11.549 - (2440.4/T_L)] \qquad (3.3)$$

Where T_L is the water temperature (K). The final value for the solubility of CO_2 in insect cell culture medium can be estimated as 25.0 mm Hg/mM, by assuming the medium ionic strength as 0.35 M (corresponding to a typical osmolarity of 0.35 M for insect cell culture medium) (Onda et al., 1970)

HCO_3^- and the base (e.g., KOH) added to control extracellular pH (pH_e) contributes to elevated medium osmolality. In mammalian cells an increase in osmolality altered cell growth and product formation (Kimura and Miller, 1996; Zhu et al., 2005). Insect cell lines are generally insensitive to medium osmolality ranging from 250 to 400 mOsm/kg (Gardiner et al., 1976). Hence, increased osmolality is unlikely to inhibit insect cell growth.

The CO_2 concentration that is typically required for normal cell growth is 30-50 mm Hg, whereas CO_2 concentrations above 100 mm Hg inhibit cell growth and productivity (Gray *et al.,* 1996). Numerous investigations conducted on CHO, hybridoma and BHK cells demonstrated that CO_2 accumulation decreases cell growth, viability and product formation (Gray *et al.,* 1996; Kimura and Miller, 1996; deZengotita *et al.,* 1998; Zhu *et al.,* 2005). Experiments conducted on CHO cells using a 10 L bioreactor demonstrated that increasing the CO_2 concentration from 36 to 145 mm Hg (osmolality – 310 to 340 mOsm/kg) decreased the maximum cell density (from 8.9 x 10^6 to 5.9 x 10^6 cell/mL), viability (from 79% to 58%) and the final productivity (by 69%) (Gray *et al.,* 1996). Similarly, an increase in CO_2 concentration from 53 to 167 mm Hg decreased the CHO cell growth by 41% (Drapeau *et al.,* 1990), whereas an increase in CO_2 concentration from 36 to 250 mm Hg (at a controlled osmolality of 310 mOsm/kg) decreased the CHO cell growth by 30% (Kimura and Miller, 1996).

Several investigations have been conducted in mammalian cells to determine the combined effect of elevated CO_2 and osmolality on cell growth and productivity (Kimura and Miller, 1996; deZengotita *et al.,* 1998; Zhu *et al.,* 2005). CHO cell growth was drastically reduced with the combined increase of CO_2 and osmolality (Table 1). However, conflicting arguments have been made on the combined effect of CO_2 and osmolality on final product concentration (Oyaas *et al.,* 1994; Reddy an Miller, 1994; deZengotita *et al.,* 2002; Kim and Lee, 2002; Zhu *et al.,* 2005). Some investigations demonstrated that elevated CO_2 concentration and osmolality increases the final product concentration, which is due to the increase of product formation in hybridoma cells under stressed conditions, e.g., elevated osmolality, whereas other investigations demonstrated

that elevated CO_2 concentration and osmolality decreases the final product concentration in other cell types, e.g., CHO cells, which is due to the decreased cell growth and viability at elevated CO_2.

In baculovirus infected Sf-9 cells the quantity of recombinant protein produced in a 150 L bioreactor was only 30% of that produced in the 70 mL spinner flask and this was believed to be due to CO_2 accumulation (Garnier *et al.,* 1996). However, no actual measurements of CO_2 were taken, but a mathematical model predicted that CO_2 levels reached a peak value of 114 mm Hg. Also, experiments were conducted in 100 mL spinner flasks to compare the recombinant protein production levels at 15% CO_2 and 0% CO_2. At 15% CO_2, the production of β-gal and TGFβ recombinant proteins reduced by 39% and 56%, respectively, compared to the production at 0% CO_2. In addition, extended protein production occurred in the 15% CO_2 environment due to slower cell death and this was hypothesized due to the inhibition of cell metabolism leading to a delayed infection process.

Accumulation of CO_2 has been shown to increase H^+ ion concentration in the cell cytoplasm, thereby leading to decreased intracellular pH (pH_i) (deZengotita *et al*., 2001; Taticek *et al*., 1998 and Adler *et al*., 1965). Changes in pH_i have detrimental effects on cells, e.g., altered cellular metabolism, enzyme activity and cell proliferation (Madshus *et al*., 1988). The increase in CO_2 concentration from 35 to 270 mm Hg in BHK-21 cells, maintained at pH_e of 6.8, decreased the pH_i from 7.2 to 6.92 (Taticek *et al*., 1998). In contrast, increasing in CO_2 concentration above 140 mm Hg did not reduce pH_i in hybridoma cells (deZengotita *et al*., 2001).

CO₂ accumulation can induce oxidative stress due to the reaction of HCO_3^- (produced from the dissociation of CO_2) with H_2O_2 (produced from the viral infection of cells), to produce carbonate anion ($CO_3^{\bullet -}$) and peroxymonocarbonate anion (HCO_4^-) (eq. 1.2 – 1.5). $CO_3^{\bullet -}$ and HCO_4^- are strong oxidizing components that can damage proteins and lipids, thereby altering cell metabolism (Goss *et al.,* 1999; Vesela and Wilhelm, 2002). Since the presence of significant levels of H_2O_2 occurs only in the presence of viral infection, it is hypothesized that CO_2 accumulation only enhances oxidative stress in infected cells.

Several different techniques have been investigated to overcome CO_2 accumulation, including (1) the replacement of bicarbonate buffer with MOPS-histidine buffer, and $NaHCO_3$ with Na_2CO_3 for pH control (Matanguihan *et al.,* 2002; Goudar *et al.,* 2006) and (2) the increase of bubble size, sparge flow rates, and by changing the impeller position (Mostafa and Gu, 2003; Gray *et al.,* 1996). Alternatively, cells can be adapted to grow in higher CO_2 concentrations (Croughan, 2009).

In a 1.5 L bioreactor, purging the headspace with air reduced the dissolved CO_2 concentration from 600 to 75 mm Hg and increased the growth rates and maximum viable cell densities of Sf-9 and Tn-5 insect cell lines (Mitchell-Logean and Murhammer, 1997). Headspace purging reduced the mean Population Doubling Times (PDT) of Sf-9 and Tn-5 cells from 38 to 22 h and from 46 to 22 h, respectively. The maximum viable cell densities increased from ~5-6 to ~8-10 x 10^6 and from ~3-5 to ~8-10 x 10^6 cells/mL in Sf-9 and Tn-5 cells, respectively. However, the CO_2 measurements taken may not have been precise since some CO_2 may have volatilized during the transfer of sample from the bioreactor for offline measurement.

The current research work studied the effect of specific CO_2 concentrations on uninfected insect cell growth and metabolism. Also, the mechanism of CO_2 inhibition in uninfected insect cells was studied by investigating the role of oxidative stress. In addition, the calibration method used to investigate the effect of elevated CO_2 on pH_i in insect cells was also discussed. Some of the limitations in previous research work, such as offline measurement of CO_2 using a blood gas analyzer or by estimating the CO_2 concentration based on mass transfer equations, have been overcome by conducting all experiments in a well-controlled bioreactor equipped with an autoclavable CO_2 probe.

3.2 Materials and Methods

3.2.1 Cell Line and Medium

The *Spodoptera frugiperda* Sf-9 insect cell line (Invitrogen, Carlsbad, CA), a clonal isolate of the IPLB-SF21-AE cell line that was isolated from the pupal ovarian tissue of the fall armyworm (Vaughn *et al.*, 1977), were grown in Sf-900 II SFM (Invitrogen, Carlsbad, CA). No additives, e.g., anti-foaming or osmolality compensating agents, were added to the cell culture medium. All chemicals were purchased from Fisher Scientific (Hampton, NH), unless specified. Cells were initially grown in 25 cm^2 T-flasks (4 mL working volume) and were used to seed 125 mL shaker flasks (20 mL working volume) at a cell density of 0.8 x 10^6 cells/mL. An orbital shaker (Thermo Scientific, Waltham, MA), maintained at a temperature of 27ºC and 140 rpm, was used to grow the cells in shaker flasks. The cells from 125 mL shaker flasks were further scaled up to a 500 mL shaker flask (80 mL working volume) and finally used to seed a 3 L bioreactor (1.2 L working volume).

3.2.2 Bioreactor

All experiments were conducted in a 3 L water-jacketed bioreactor (Broadley-James, Irvine, CA) with a working volume of 1.2 L (Figure 4, 5). The bioreactor was fitted with a scoping marine impeller and the agitation was maintained at 250 rpm. The bioreactor temperature was maintained at 27ºC using a water bath (Thermo Scientific, Waltham, MA). The dissolved oxygen concentration was measured by means of a polarographic oxygen probe (Mettler-Toledo, Columbus, OH) and was controlled at 20% air saturation by sparging pure oxygen when required using a DO controller (B&C Electronics, Milano, Italy) and a solenoid valve (Burkert, Ingelfingen, Germany). The final gas sparge flow rate through the cell culture medium was limited to 0.02 - 0.06 vvm. The pH was measured using a pH probe (Phoenix Electrodes, Houston, TX) and was controlled at 6.2 ± 0.1 by manually adding 0.5 M KOH using a peristaltic pump (Cole-Parmer, Vernon Hills, IL). In order to prevent CO_2 accumulation within the bioreactor, the headspace was continuously purged with air at a flow rate of 200 mL/min. The CO_2 concentration in the cell culture medium was monitored on-line using YSI 8505 autoclavable CO_2 probe with YSI 8500 CO_2 monitor (Yellow Springs Instruments, Yellow Springs, OH). The CO_2 concentration within the bioreactor was manually controlled at the desired concentration using rotometers.
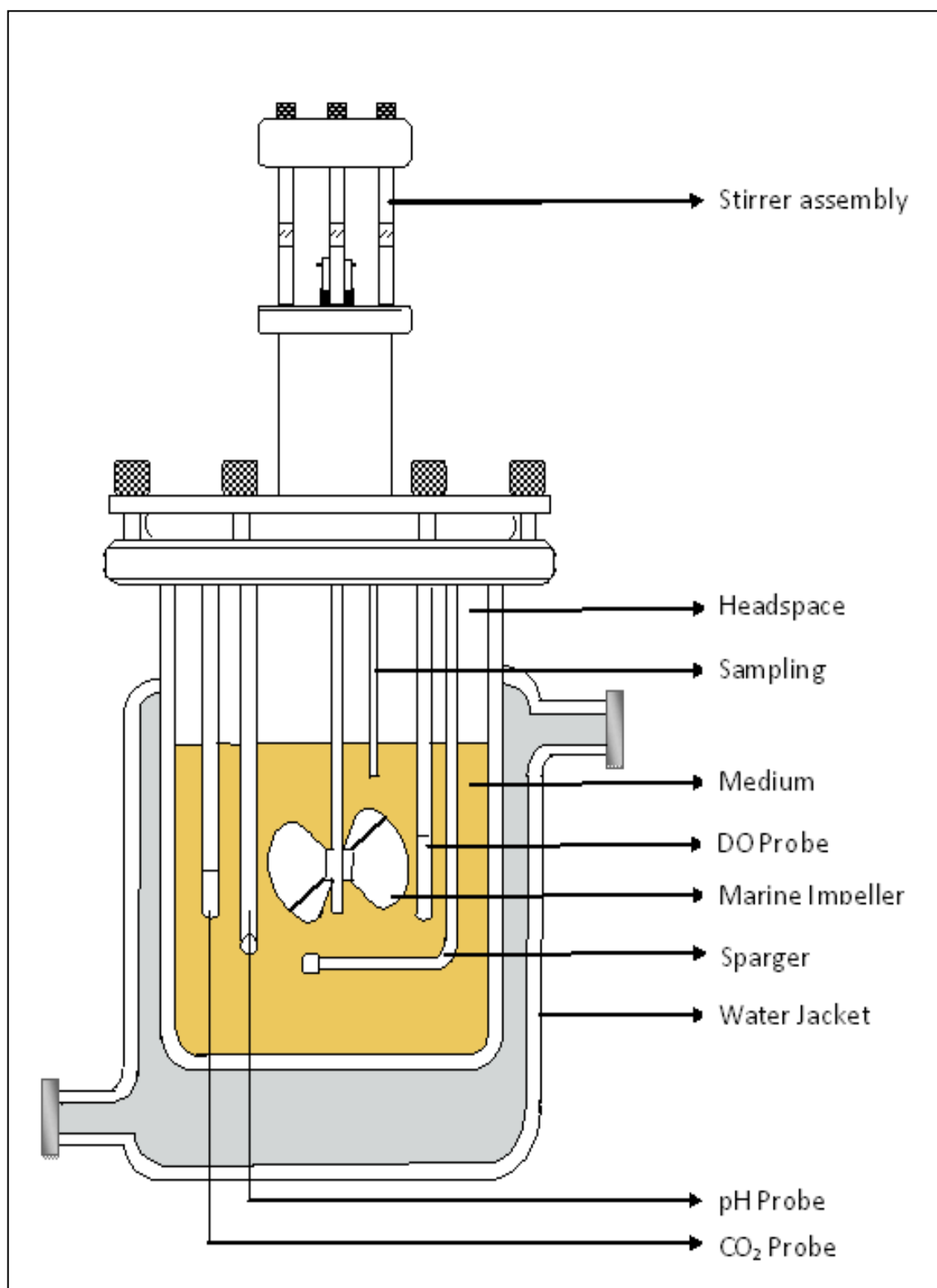
Figure 3: Schematic of water-jacketed bioreactor equipped with pH, dissolved oxygen and CO_2 probes
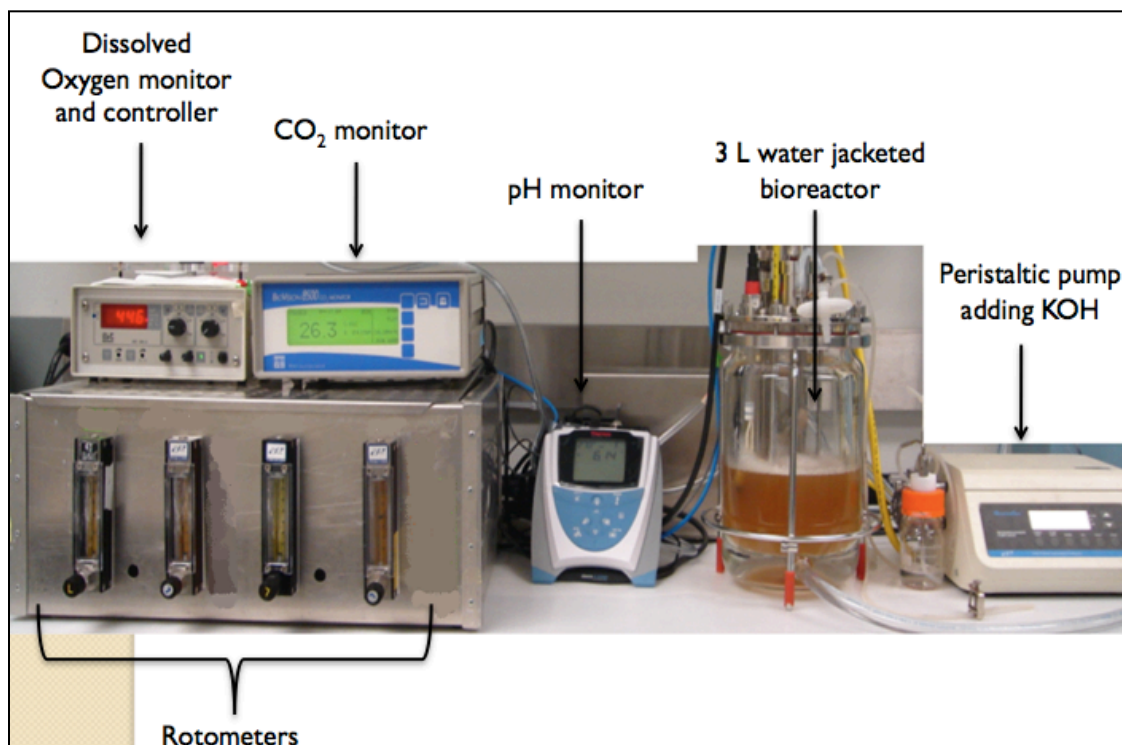
Figure 4: Experimental set-up

The bioreactor was seeded with Sf-9 cells at a density of 0.8×10^6 cells/mL and viability over 90%. During the lag phase (0-24 h of inoculation) the CO_2 concentration within the bioreactor was maintained below 37 mm Hg by continuously purging the medium and the headspace with air. After 24 h of inoculation, during which the cells were usually in exponential growth phase, a constant CO_2 concentration (73, 147, 183 or 220 mm Hg, i.e., an aqueous phase concentration in equilibrium with a gas phase with the CO_2 partial pressure of 73, 147, 183 or 220 mm Hg) was maintained within the bioreactor by purging the medium with the required CO_2 and the headspace with air. The CO_2 concentration was maintained in the range of ±15 mm Hg of the stated value.

A control experiment, i.e., using a bioreactor maintained at the lowest possible CO_2 concentration (0 – 37 mm Hg), was conducted by purging the medium and the

headspace with air throughout the experiment (even during the exponential growth phase). Also, cells were grown in 500 mL shaker flasks (80 mL working volume) to compare the growth and metabolism with cells grown in the bioreactor at different CO_2 concentrations. The experiments were repeated several times and samples were collected every 12 - 24 h. Cell density, cell viability and osmolality were immediately measured.

3.2.3 Analytical

The samples collected every 12 – 24 h were centrifuged at 1000 x g for 5 min to separate the supernatant from the cell pellet. The supernatant was stored at –20ºC and was used to measure glucose and lactate concentrations. The resulting cell pellet was washed 2-3 times with Dulbecco's potassium phosphate buffered saline (1X PBS) and stored in –80ºC freezer until assayed. Prior to assaying, the samples were removed from the –80ºC freezer, quickly thawed and resuspended in PBS solution. The thawed cells were kept on ice and were then lysed twice for 5 s using a sonicator operated at 20% full power (Misonix, Farmingdale, NY). The resulting homogenate was used for the lipid hydroperoxide assay after centrifuging at 3000 x g for 5 min at 4ºC and protein carbonyl assay after centrifuging at 16,000 x g for 5 min at room temperature.

Cell density was measured using a Vi-Cell counter (Beckman Coulter, Crea, CA) and cell viability was measured by trypan blue exclusion method using a hemocytometer (Nikon, Melville, NY). The specific growth rate (µ) of Sf-9 cells grown at a given CO_2 concentration was obtained from the slope of the logarithm of viable cell density in exponential growth phase region versus time. This µ was used to find the Population Doubling Times (PDT) of Sf-9 cells cultivated at a given CO_2 concentration. The PDT values were reported based on 95% confidence levels, calculated using the student's t-test

method (Shoemaker *et al.,* 1974). The osmolality of the cell culture medium was measured using a freezing point osmometer (Advanced Instruments Inc., Norwood, MA).

Glucose and lactate concentrations in the cell culture medium were measured using a YSI 2300 glucose and lactate analyzer (Yellow Springs Instruments, Yellow Springs, OH). The resulting data were plotted against the corresponding cell density in exponential growth phase. The specific glucose consumption/lactate production rates were estimated from the ratio of the specific growth rate and the slope of glucose/lactate concentration vs cell density plot (Rhiel *et al*., 1997).

3.2.4 Assays

3.2.4.1 Lipid Hydroperoxide Assay

Lipid hydroperoxide concentration was determined by using the BIOXYTECH® LPO-586 assay kit (OXIS International Inc., Beverly Hills, CA), following the manufacturer's instructions. This assay is based on the reaction of malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) (produced due to the decomposition of unstable lipid peroxides) with N-methyl-2-phenylindole in the presence of a methanesulfonic acid. The resultant product was quantified by determining absorbance at 586 nm using a spectrometer (Thermo Scientific, Waltham, MA). Initially, a peroxide calibration plot was developed using the absorbance of different peroxide concentrations at 586 nm by using the standard solution provided by the manufacturer. This calibration curve was used to determine the peroxide concentration present in the sample. The total protein concentration present in the sample was determined by using the Bradford assay. Different dilutions of Bovine Serum Albumin (BSA) (Sigma-Aldrich, St. Louis, MO) in H_2O (0–1 mg/mL) were mixed with the reagent dye and the absorbance at 595 nm was

measured. These data were used to develop a protein calibration curve from which the total protein concentration present in the sample was determined. The final lipid hydroperoxide concentration was expressed as the ratio of the number of moles of peroxides in the sample to the mass of the protein present in the sample.

3.2.4.2 Protein Carbonyl Assay

The protein carbonyl assay was based on the principle that reactive oxygen species attack amino acid residues in proteins (especially histidines, arginines, lysines, and prolines) to produce carbonyl products (Levine *et al*., 1990; Wang *et al*., 2001). These carbonyl products can be reacted with 2,4-dinitrophenylhydrazine (DNP) (Sigma-Aldrich, St. Louis, MO) to form hydrazone derivatives that can be measured spectroscopically. This assay was conducted by reacting 250 μL of cell homogenate supernatant containing approximately 1 mg/mL of protein with 250 μL of DNP in 2 M HCl (+DNP) or 250 μL of 2 M HCl (-DNP) in dark at room temperature for 15 min, with vortexing every 5 min (Hawkins *et al*., 2009). After reacting for 15 min, 125 μL of 50% trichloroacetic acid was added to the samples and then incubated at -20ºC for 15 min. After treating with trichloroacetic acid, samples were centrifuged at 9,000 x g for 15 min at room temperature. The protein pellet was washed 3 times with ethanol: ethyl acetate (1:1) by centrifuging at 9,000 x g for 2 min between the washes to remove any leftover reagent without disturbing the pellet. The resulting protein pellet was dissolved in 1 mL of 6 M guanidine-HCl solution. The samples were centrifuged at 9,000 x g for 2 mins to remove any insoluble components. The absorption of the samples treated with DNP (+DNP) was measured at 370 nm and the carbonyl content was determined by using a molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$. Guanidine-HCl solution (6 M) was used

as the blank. The total protein concentration present in the sample was measured from the samples treated with HCl (-DNP). Briefly, the absorbance of different concentrations of BSA (0-1 mg/mL) in guanidine-HCl at 280 nm was used as a calibration curve from which the sample's final protein concentration was determined. The final protein carbonyl concentration was expressed as the ratio of the number of moles of carbonyls present in the sample to the protein mass in the sample.

3.2.4.3 Intracellular pH

Intracellular pH (pH_i) of Sf-9 cells was determined using 2',7'-bis-(2-carboxyethyl)-5-(and-6—carboxyfluorescein, acetoxymethyl ester (BCECF-AM) (Invitrogen, Carlsbad, CA), a non-fluorescent ester form of the fluorescent probe BCECF attached to AM ester group (Ozkan and Mutharansan, 2002; Vachon *et al.*, 1995). The AM ester groups act as uncharged lipophilic blocking groups that insulate the charged carboxylic acid groups, thus providing cell membrane permeability. The AM ester groups of BCECF-AM are removed by cellular esterases within the cell to expose the fluorescent BCECF. BCECF is not as membrane permeable as the uncharged form, and therefore swift leakage from the cell is prevented.

The assay method involves the development of a calibration curve using nigericin (Invitrogen, Carlsbad, CA), which equilibrates the pH_i and extracellular pH (pH_e) in the presence of high potassium ion concentration. Specifically, 5×10^6 cells/mL were suspended in fresh medium maintained at different pH_e's (6.00 to 7.50, with increments of 0.25). BCECF-AM (0.5 µM), nigericin (10 µM), and KCl (150 mM) were added to the suspension and were incubated at 27ºC for 30 min. Under these conditions, measuring the pH_e with a standard pH meter (Thermo Scientific, Waltham, MA) gives

the pH_i of the cells. After incubation, the cells were centrifuged at 325 x g for 5 min and were brought back in the fresh medium maintained at respective pH_e's with no additives. The excitation intensity of wavelengths ranging from 410-515 nm were obtained in increments of 1 nm using a spectrofluorometer (Perkin Elmer Instruments, Waltham, MA), while holding the emission wavelength constant at 535 nm. The emission and excitation slit widths were both 4 nm, respectively, and the scan speed was 500 nm/s. A parabolic calibration curve was developed by plotting pH_i against the ratio of the absorption intensity at maximum absorption (usually observed at 507 nm) and the absorption intensity at isobestic point (usually observed at 424 nm). To avoid any cell-to-cell variations, dye loading differences or discrepancies in the intensities of BCECF-AM, a calibration curve has to be developed each time prior to measuring the pH_i. After developing the calibration curve, the pH_i of the sample was determined by adding 0.5 μM BCECF-AM ester to the cell suspension (5 x 10^6 cells/mL) (nigericin or potassium ions were not added and the medium pH was not corrected) and incubating for 30 min at 27ºC. The incubated sample was brought up in 1 mL fresh medium without additives after centrifuging at 325 x g for 5 min. The intensities at isobestic point (wave length at which samples have same absorption intensity) and maximum absorption were measured and were used to determine the sample pH_i from the respective calibration curve.

3.3 Results and Discussion

<u>3.3.1 Effect of CO_2 on Sf-9 Cell Growth</u>

3.3.1.1 Population Doubling Time (PDT)

The PDT of cells grown in shaker flasks (i.e., 24.9 ± 3.1 h; number of experiments, n = 7; 95% confidence levels) was similar to the PDT of cells grown in

bioreactor maintained at 0-37 mm Hg (i.e., 23.2 ± 6.7 h; n = 3). These results coincided with the results of Mitchell-Logean and Murhammer (1997), where the bioreactor headspace purging resulted in an average PDT of 22 h with Sf-9 cells. Figure 5 illustrates the typical growth curves of Sf-9 cells at different CO_2 concentrations. The increase in CO_2 concentration from 0-37 to 183 mm Hg gradually increased the PDT from 23.2 ± 6.7 to 43.0 ± 5.4 h after which an increase in CO_2 concentration to 220 mm Hg drastically increased the PDT to 69.3 ± 35.9 h (n = 4) (Figure 6). The significant error that occurred in calculating the PDT at 220 mm Hg might be due to an experimental error in one of the measurements (Table 2). An attempt to eliminate the suspected value using the Q-test method at 95% confidence level was not successful.
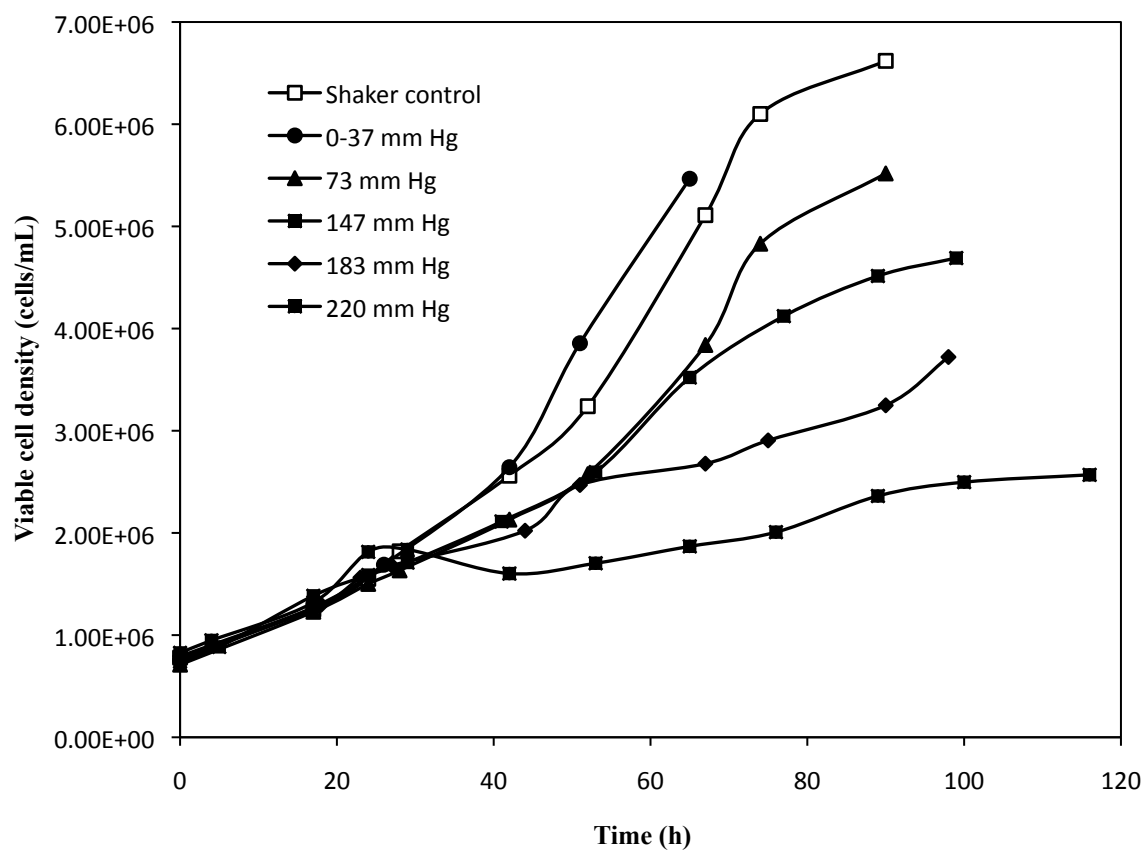

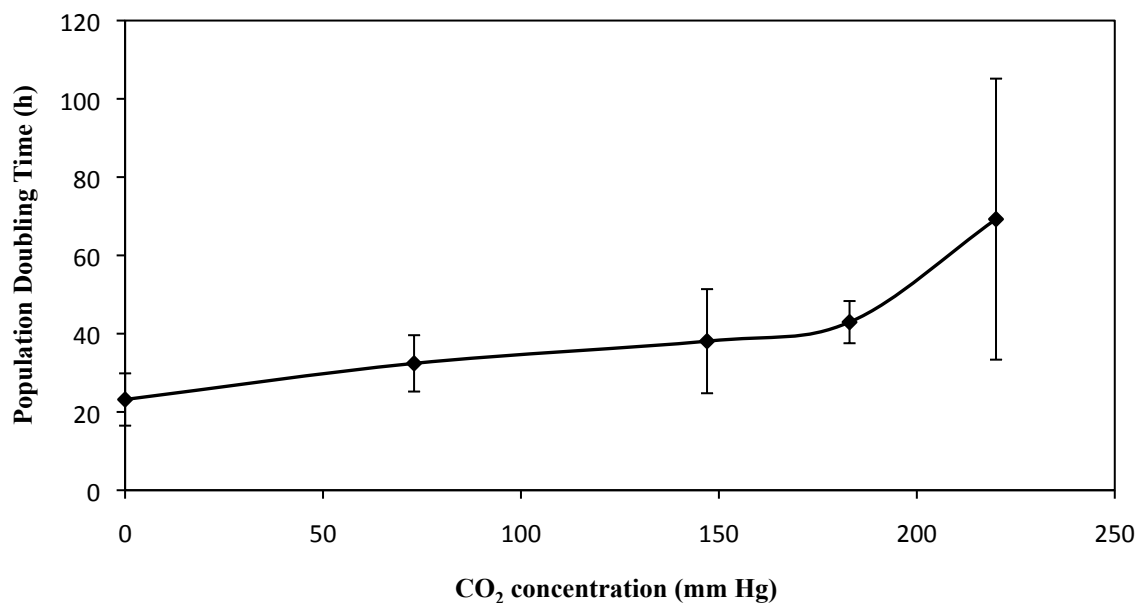Figure 5: Typical growth curves of Sf-9 cells at differenct CO_2 concentrations

Figure 6: Population Doubling Time (PDT)* of Sf-9 cells at different CO_2 concentrations

* PDT determined for n = 3 or 4 at 95% confidence levels

Table 2: Population Doubling Time of Sf-9 cells at different CO_2 concentrations

CO_2 (mm Hg)	PDT for several repetitions (h)							Mean	n	PDT[a] (h)
0-37	26.2	20.9	22.6	-	-	-	-	23.2	3	23.2 ± 6.7
73	29.9	35.6	31.8	-	-	-	-	32.4	3	32.4 ± 7.2
147	36.3	50.2	34.8	31.1	-	-	-	38.1	4	38.1 ± 13.3
183	43.6	40.5	44.7	-	-	-	-	43.0	3	43.0 ± 5.4
220	102[b]	51.0	58.7	65.4	-	-	-	69.3	4	69.3 ± 35.9
Shaker	26.0	27.6	26.8	20.9	29.5	21.8	21.8	24.9	7	24.9 ± 3.1

n-number of experiments; a-calculated using 95% confidence levels; b-suspect

3.3.1.2 Cell Viability

Increasing the CO_2 concentration did not affect Sf-9 cell viability. The viability of Sf-9 cells in all experiments was observed to be over 90%. These results are in agreement with the results of Mitchell-Logean and Murhammer (1997) and Zhu *et al*. (2005) who reported that an elevated CO_2 concentration did not affect cell viability of Sf-9 and CHO cells, respectively. In contrast, Gray *et al.* (1996) observed a 27% decrease in CHO cell viability due to an increase in CO_2 concentration from 35 to 148 mm Hg (osmolality – 310 – 340 mOsm/kg).

3.3.2 Effect of CO_2 on Medium Osmolality

A systematic decrease in osmolality, from 360 to 300 mOsm/kg, was observed in both bioreactor and shaker flask experiments. This range is known to have a negligible effect on insect cell cultures (Gardiner *et al.*, 1976); thus, the reduced Sf-9 cell growth is likely to be primarily due to CO_2 inhibition rather than altered medium osmolality. The decreased osmolality trend did not change with different CO_2 concentrations and hence an increase in CO_2 concentration does not significantly affect the osmolality of Sf-9 cell culture medium.

3.3.3 Effect of CO_2 on Sf-9 Cell Metabolism

3.3.3.1 Glucose Consumption

The glucose concentrations of Sf-9 cells grown in shaker flasks and in the bioreactor controlled at different CO_2 concentrations were measured (Figure 7). It was observed that the specific glucose consumption rate of Sf-9 cells exposed to different CO_2 concentrations did not vary significantly (Table 3). Also, the glucose consumption rate of Sf-9 cells grown in shaker flasks (2.6×10^{-17} mol/cell-s) was similar to the glucose

consumption rate of Sf-9 cells grown in the bioreactor at different CO_2 concentrations $(2.5 - 3.2 \times 10^{-17}$ mol/cell-s). These results are consistent with those of Rhiel *et al.* (1997), who reported the specific glucose consumption rate of uninfected Sf-9 cells as 2.4×10^{-17} mol/cell-s.
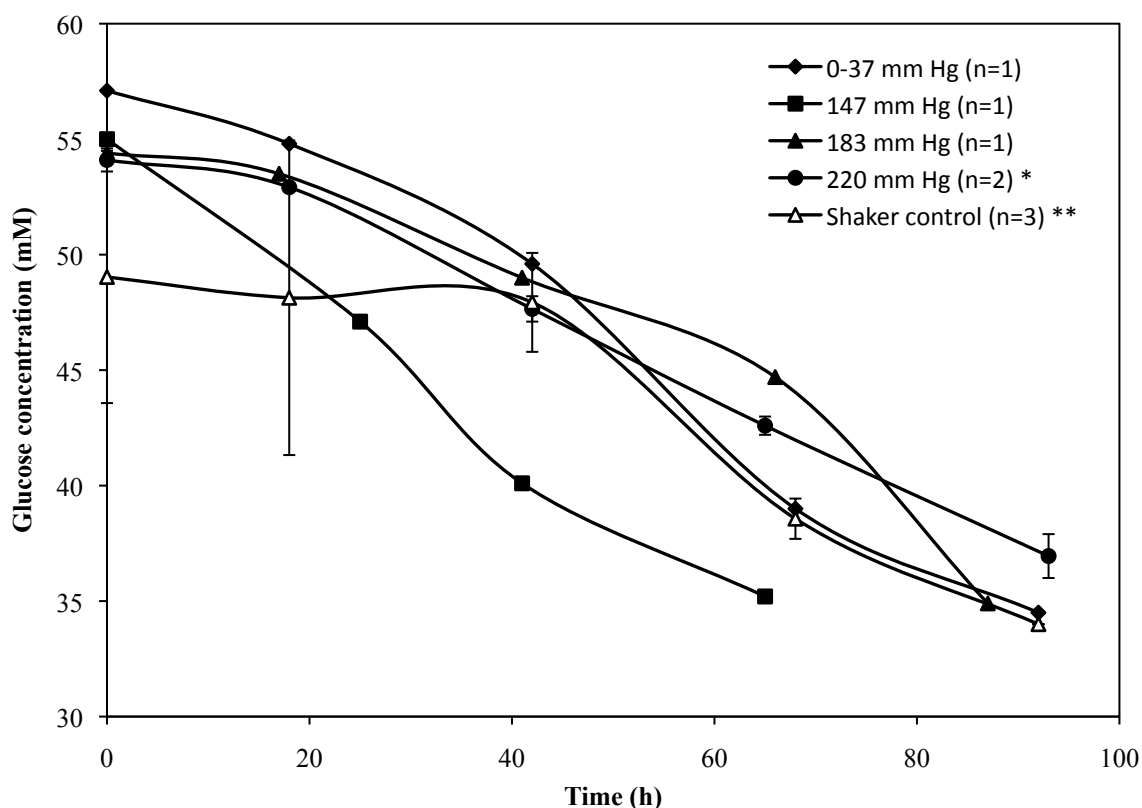

Figure 7: Glucose concentration of Sf-9 cell culture in shaker flask and in bioreactor controlled at different CO_2 concentrations

* range; ** 95% confidence levels

3.3.3.2 Lactate Production

The lactate concentrations of Sf-9 cells grown in shaker flask and in bioreactor exposed to different CO_2 concentrations were calculated (Table 3). Unlike Tn-5 cells, which produce lactate at a rate of 5.8×10^{-17} and 2.6×10^{-17} mol/cell-s in uninfected and infected cells, respectively, Sf-9 cells do not produce lactate under physiological

conditions (Rhiel *et al.*, 1997). However, a noticeable increase in specific lactate production was observed in uninfected Sf-9 cells with the increase in CO_2 concentration (Figure 8). Cells grown in shaker flasks and cells grown in the bioreactor controlled at 0–37 mm Hg CO_2 consumed the lactate that was initially present in the cell culture medium and no lactate was produced under these conditions. In contrast, lactate production increased from -3.0 x 10^{-19} to 10.2 x 10^{-19} mol/cell-s with the increase in CO_2 concentration from 0-37 to 220 mm Hg (Figure 9). These results demonstrated that the exposure of Sf-9 cells to CO_2 created a stressed environment and thus induced lactate production.
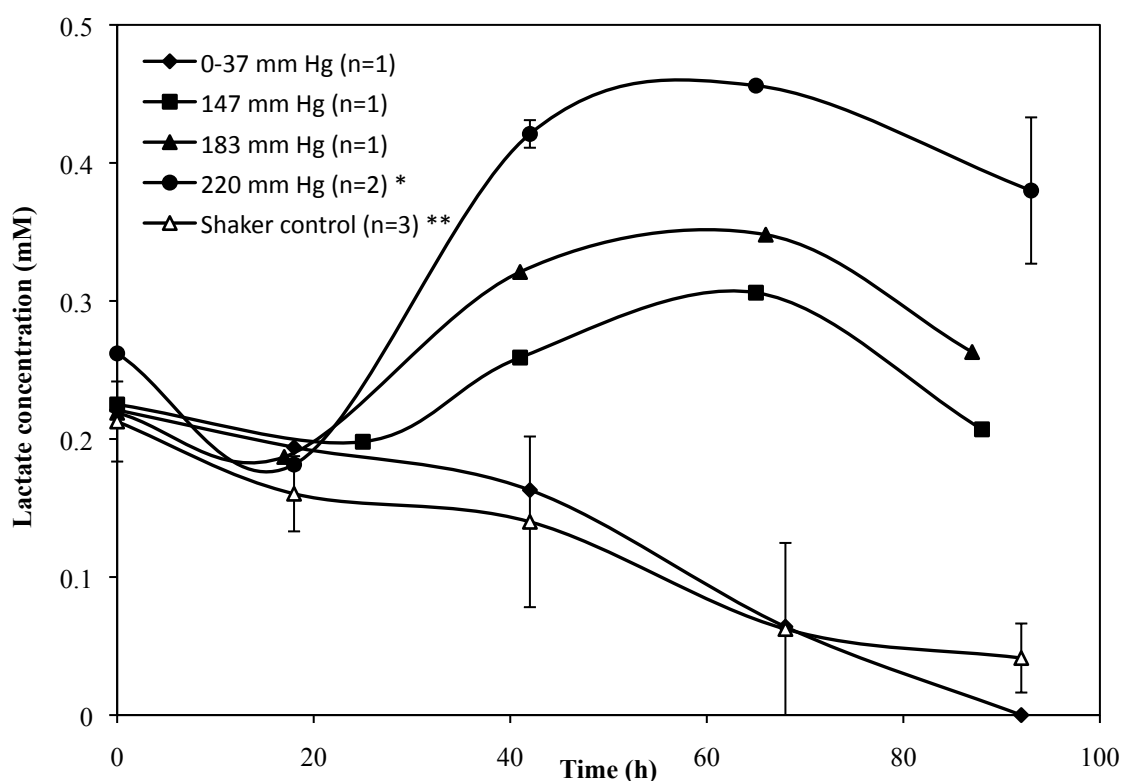

Figure 8: Lactate concentration of Sf-9 cell culture in shaker flask and in bioreactor controlled at different CO_2 concentrations
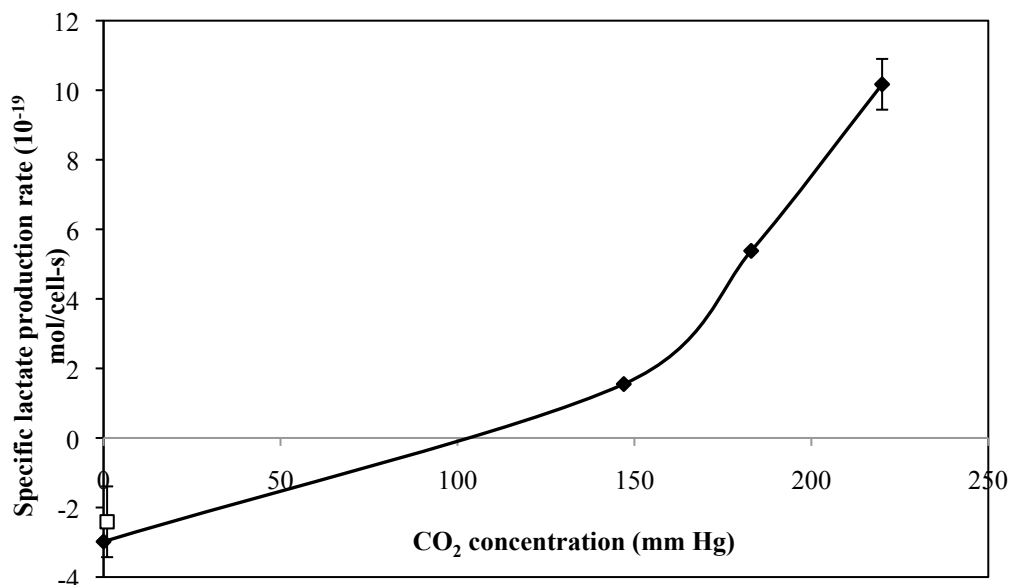
* range; ** 95% confidence levels

Figure 9: Specific lactate production rate of Sf-9 cells at different CO_2 concentrations*

□ represents the shaker flask experiments (n = 3) at 95% confidence levels

* n = 1 at 0 mm Hg, 147 mm Hg and 183 mm Hg; n = 2 at 220 mm Hg and the range was indicated

Table 3: Glucose production and lactate consumption rates of Sf-9 cells at different CO_2 concentrations

CO_2 (mm Hg)	No. of experiments (n)	Glucose conc. (10^{-17} mol/cell-s)	Lactate conc. (10^{-19} mol/cell-s)
0-37	1	-3.2 [a]	-3.0
73	0	-	-
147	1	-2.9	1.6
183	1	-2.5	5.4
220	2	-2.9 ± 0.1 [b]	10.2 ± 1.5 [b]
Shaker flask	3	-2.6 ± 1.6 [c]	-2.4 ± 1.0 [c]

a-negative sign indicates substrate consumption; b-range; c-95% confidence levels

3.3.4 Effect of CO_2 on Oxidative Stress

The lipid hydroperoxide concentration and protein carbonyl concentration of Sf-9 cells grown in shaker flasks and in the bioreactor at different CO_2 concentrations was measured to determine the role of oxidative stress in CO_2 inhibition. Negligible levels of lipid peroxides were detected in shaker flask and in bioreactor experiments. Also, it was discovered that the lipid peroxide concentrations detected in uninfected Sf-9 cells did not change with elevated CO_2 concentrations (Figure 10). Similarly, the results of protein carbonyl assay indicated that elevated CO_2 concentration did not affect the carbonyl concentration in uninfected Sf-9 cells (Figure 11).
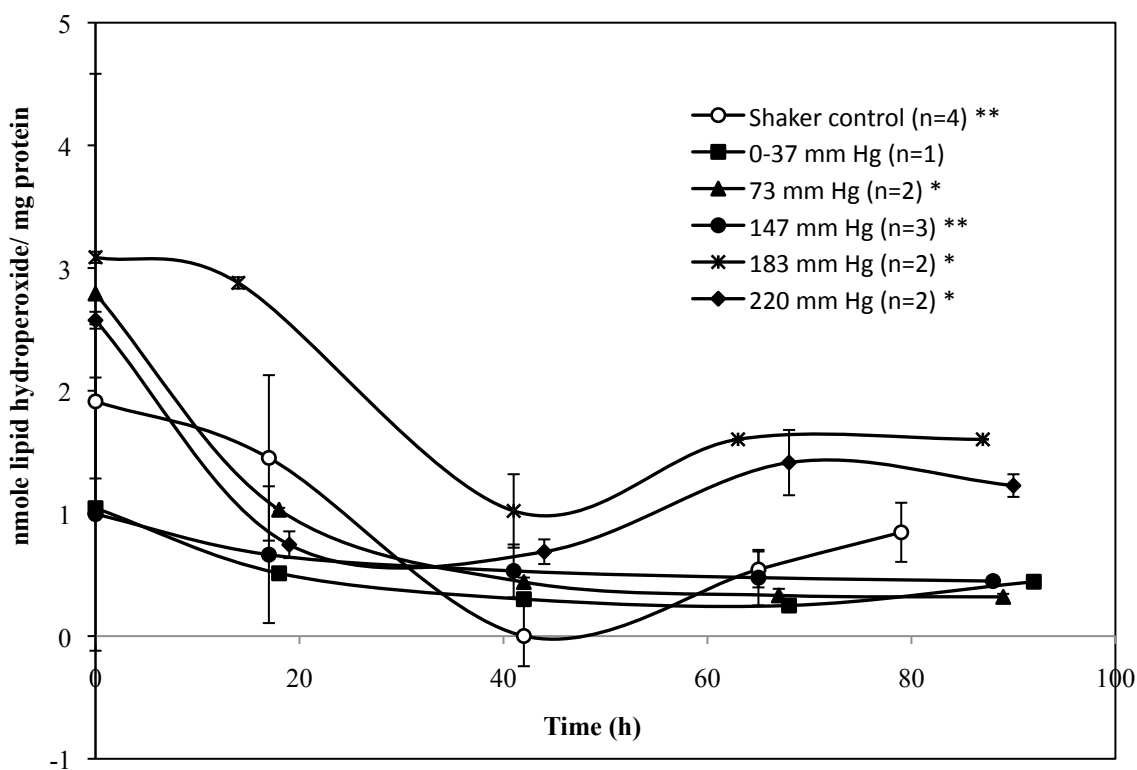

Figure 10: Lipid hydroperoxide concentrations of Sf-9 cells grown in shaker flask and in bioreactor controlled at different CO_2 concentrations
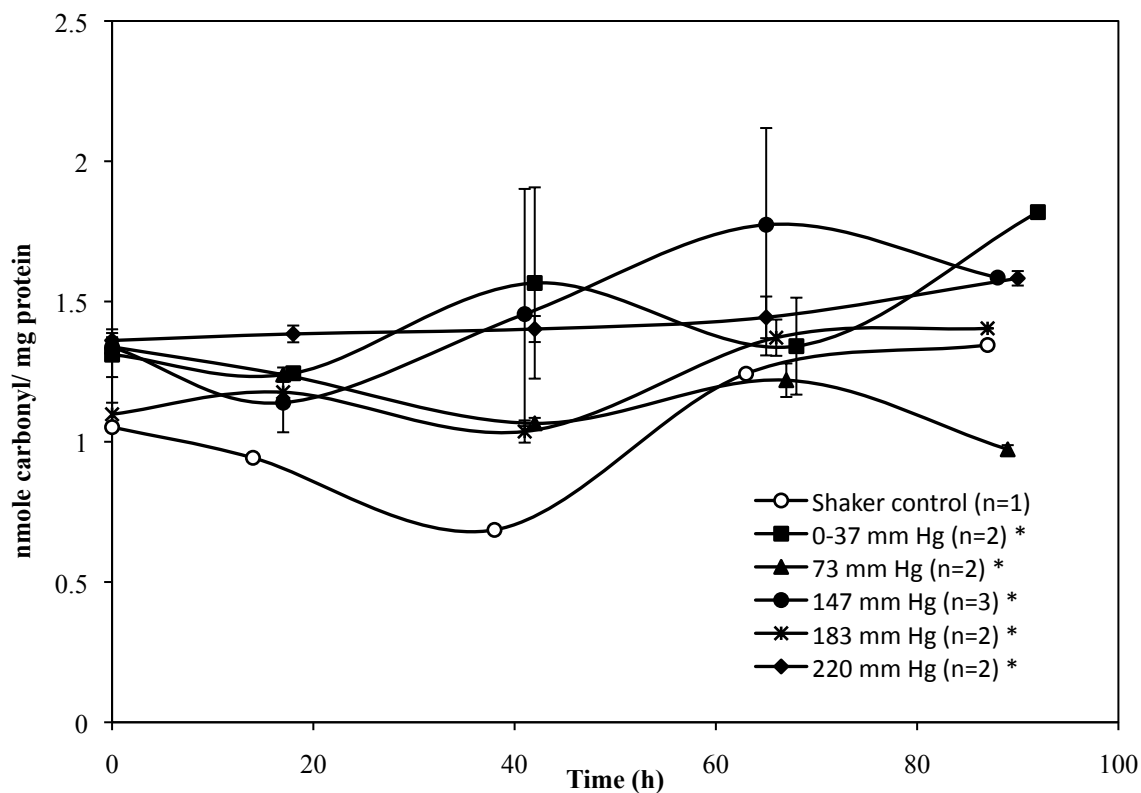
* range; ** 95% confidence levels

Figure 11: Protein carbonyl concentrations of Sf-9 cells growth in shaker flask and in bioreactor controlled at different CO_2 concentrations

* range

3.3.5 Effect of CO_2 on Intracellular pH (pH_i)

Experiments were conducted to develop the calibration curve and to determine the pH_i of Sf-9 cells. An independent calibration curve was developed each time prior to the measurement of the pH_i of the given sample. The typical excitation scans for the calibration data and the typical calibration curves were shown in Figure 12 and 13, respectively. Further experiments involving the changes in pH_i caused due to elevated CO_2 concentrations has to be conducted. Such experiments should be conducted with extreme caution to prevent the escape of CO_2 from the sample culture medium while performing the pH_i assay.
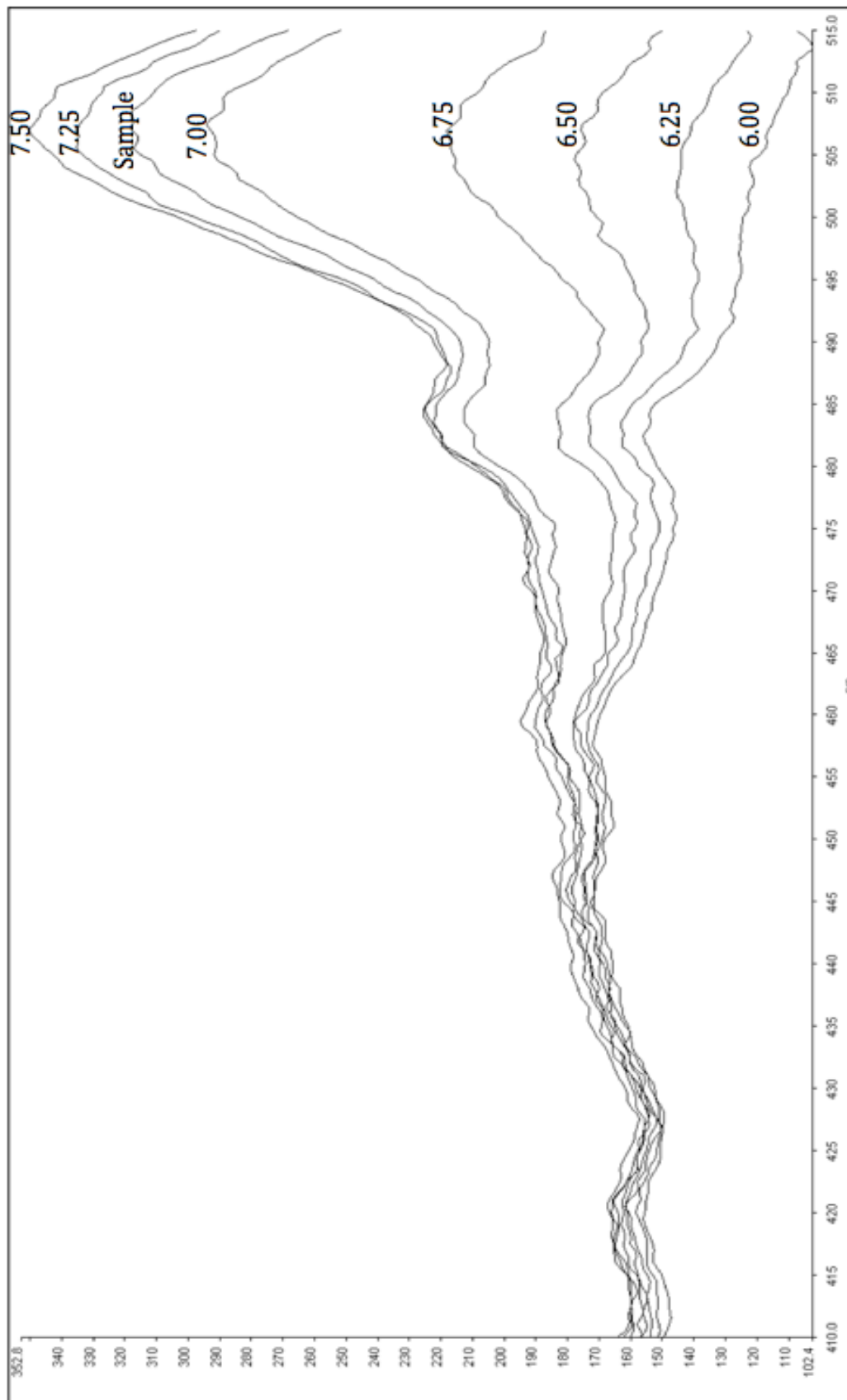
Figure 12: Typical excitation scans (410 to 515 nm) of uninfected Sf-9 cells and Sf-9 cells during calibration*

* Calibration pH range from 6.00 to 7.25; Sample set indicates the cells that were not treated with nigericin
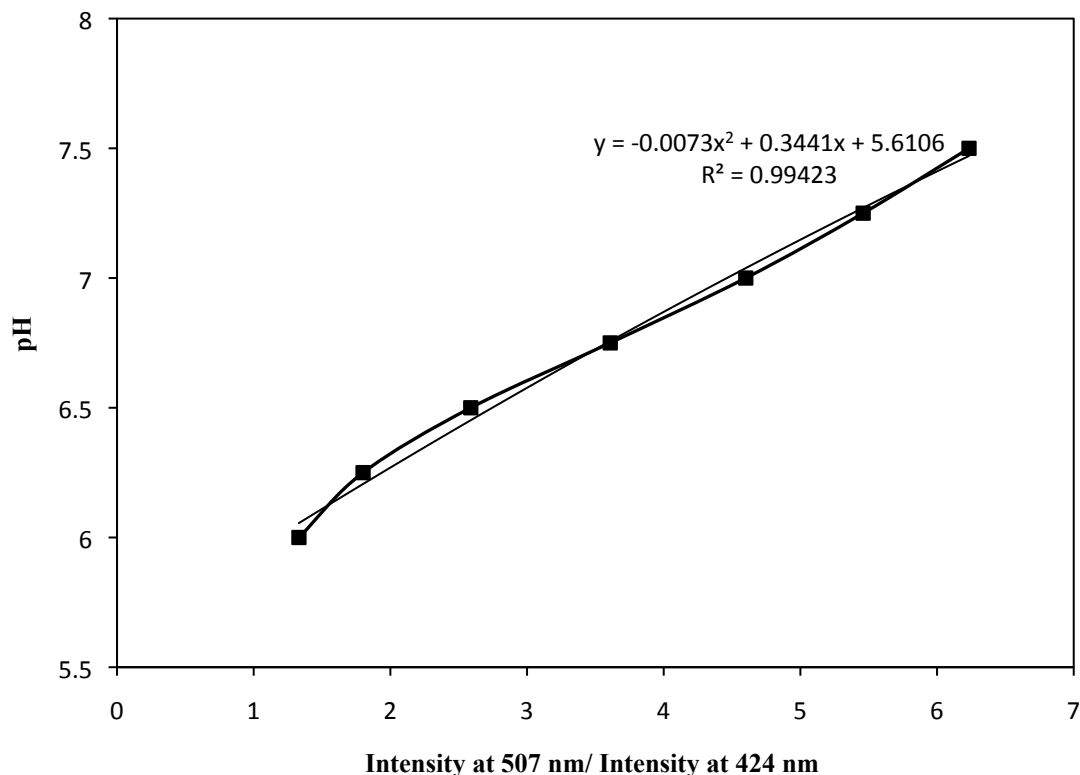
Figure 13: Typical calibration curve for pH_i

3.4 Conclusions

The increase in CO_2 concentration resulted in reduced cell growth and increased PDT in uninfected Sf-9 cells. Elevated CO_2 concentration did not significantly alter cell viability, medium osmolality and the specific glucose consumption rate. However, increased CO_2 concentrations resulted in increased lactate production in uninfected Sf-9 cells. Since no significant levels of lipid hydroperoxide and protein carbonyl concentrations were detected, it could be concluded that oxidative stress did not contribute to CO_2 inhibition in uninfected Sf-9 cells. Future work related to the role of pH_i in CO_2 inhibition in uninfected Sf-9 cells will yield useful information in finding the mechanism behind CO_2 inhibition.

CHAPTER – 4

CONCLUSIONS AND FUTURE WORK

It was demonstrated that CO_2 accumulation inhibits the growth of uninfected Sf-9 cells, but does not affect cell viability. Furthermore, the specific glucose consumption rate was not affected by increasing CO_2 concentrations, but lactate production increased slightly with increasing CO_2 levels. Oxidative stress was demonstrated not to be involved in CO_2 inhibition. Finally, evidence supports the premise that the mechanism of CO_2 inhibition is due to intracellular acidification.

Future investigations concerning the effect of CO_2 in intracellular acidification of uninfected insect cells are necessary. Furthermore, studies involving the effect of elevated CO_2 concentration on baculovirus infected insect cell growth and productivity need to be conducted. Since oxidative stress and pH_i are believed to be the potential causes for reduced productivity in baculovirus infected insect cells, such investigations are necessary and can yield valuable facts to develop methods to overcome CO_2 inhibition in large-scale bioreactors. Specifically, investigations related to the genetic engineering of insect cells to express high antioxidant enzymes, e.g., superoxide dismutase, catalase etc., to counteract the oxidative stress or antiporters to counteract the pH_i caused due to CO_2 accumulation will be useful in developing techniques to counteract CO_2 inhibitory effect.

APPENDIX A

GOWTH CURVES OF Sf-9 CELLS AT DIFFERENT CO_2 CONCENTRATIONS
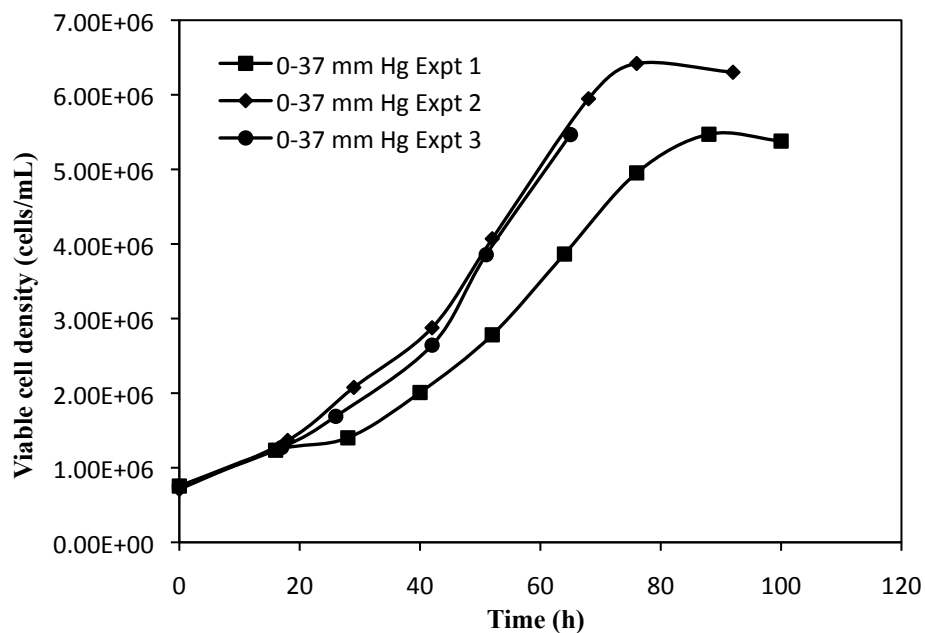
Figure A1: Growth curves of Sf-9 cells exposed to 0-37 mm Hg CO_2
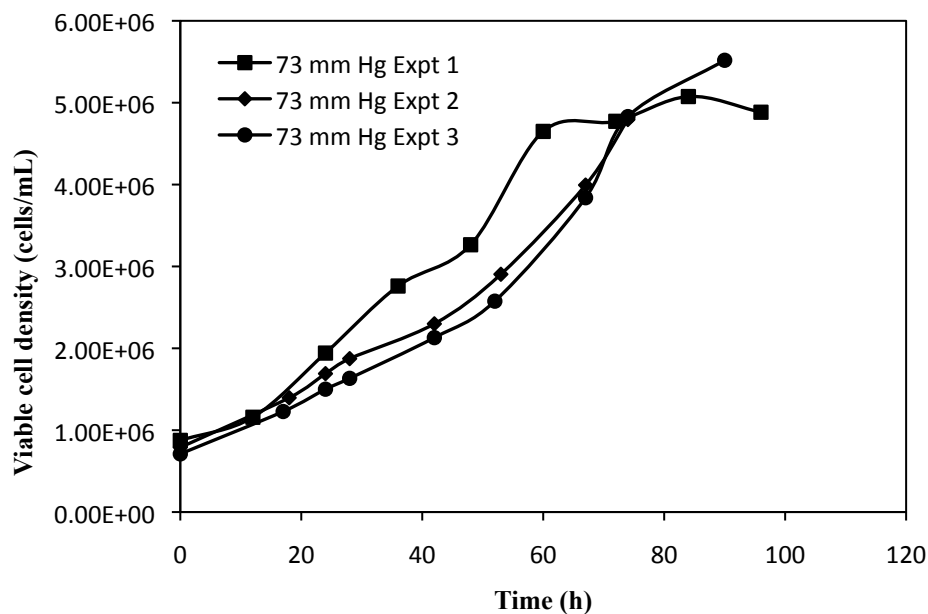
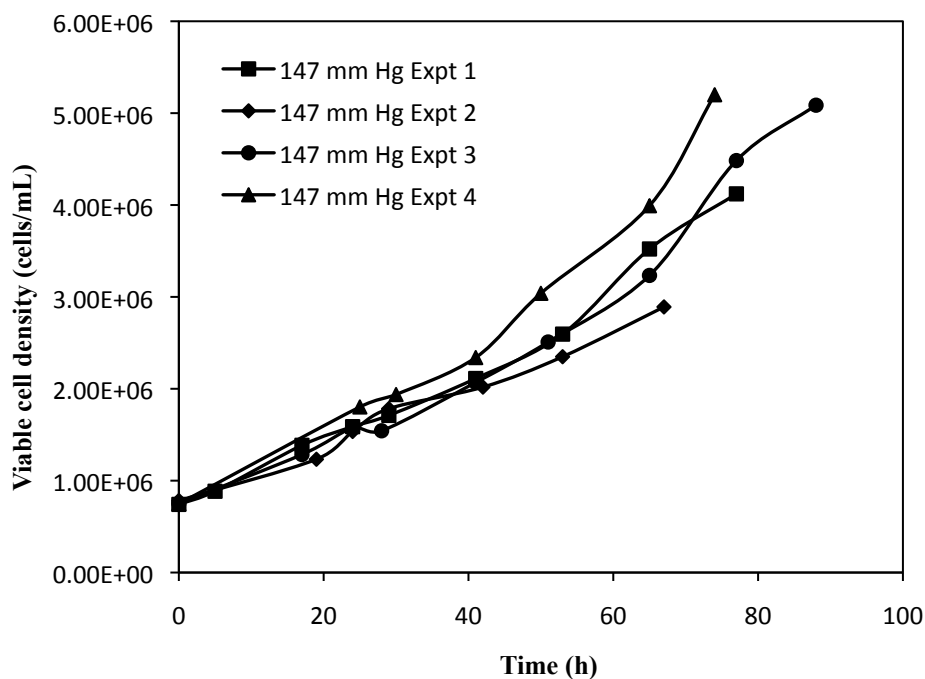Figure A2: Growth curves of Sf-9 cells exposed to 73 mm Hg CO_2

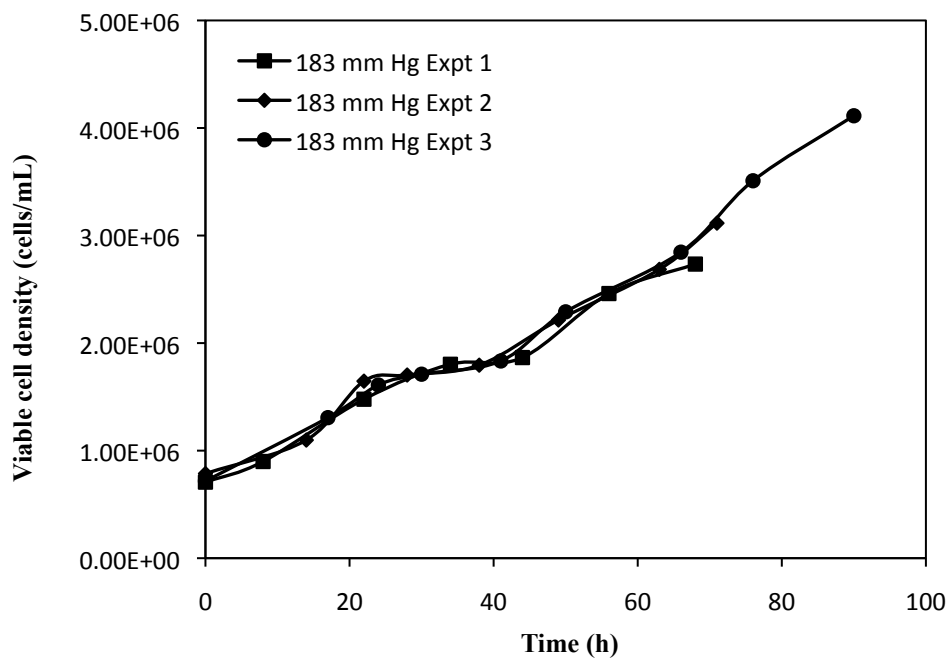Figure A3: Growth curves of Sf-9 cells exposed to 147 mm Hg CO_2

Figure A4: Growth curves of Sf-9 cells exposed to 183 mm Hg CO_2
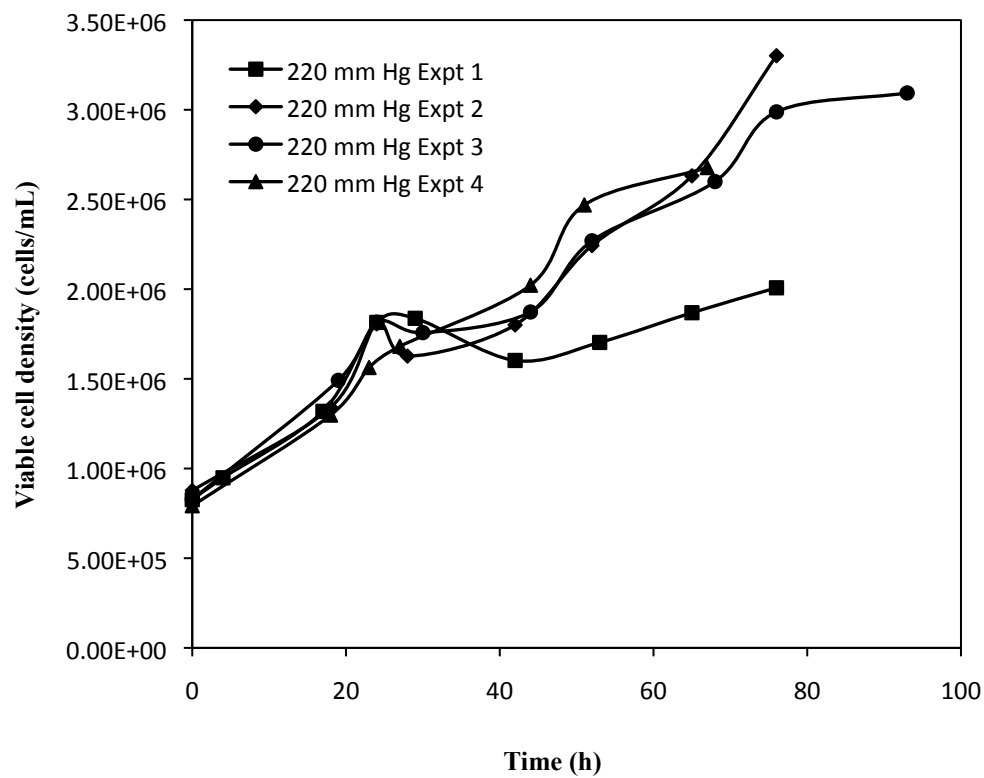
Figure A5: Growth curves of Sf-9 cells exposed to 220 mm Hg CO_2

REFERENCES

Adler, S.; Roy, A.; Relman, S. Intracellular acid-base regulation. II The interaction between CO_2 tension and extracellular bicarbonate in the determination of muscle cell pH. *J. Clin. Invest.***1965**, 44, 21-30.

Agathos, N.S. Insect cell bioreactors. *Cytotechnol*. **1996,** 20, 173-189.

Asenjo, J.A. *Bioreactor system design*; Marcel Dekker Inc.: New York, 1995.

Aunins, J.G.; Henzler, H-J. *Aeration in cell culture bioreactors*; Weinheim: VCH Verlag, 1993.

Chang, G.D.; Chen, C-J.; Lin, C-Y.; Chen, H-C.; Chen, H. Improvement of glycosylation in insect cells with mammalian glycosyltransferases. *J. Biotechnol.* **2003**, 102, 61-71.

Croughan, M. In *Evolution in biotechnology: cells, processes and organizational structures*. Presented at 18th Biocatalysis and Bioprocessing Conference. Iowa City, IA, 2009.

deZengotita, V.M.; Kimura, G.; Miller, W.M. Effects of CO_2 and osmolality on hybridoma cells: Growth, metabolism and monoclonal anti-body production. *Cytotechnol.* **1998**, 28, 213-227.

deZengotita, V.M.; Schmeizer, A.E.; Miller, W.M. Characterization of hybridoma cell response to elevated pCO_2 and osmolality: Intracellular pH, cell size, apoptosis and metabolis. *Biotechnol. Bioeng.* **2002**, 77, 369-380.

Donaldson, M.S., Shuler, M.L. Low-cost serum free medium for the BTI-Tn5B1-4 insect cell line. *Biotechnol. Prog.* **1998**, 14, 573-379.

Drapeau, S.L.; Luan, Y-T.; Whiteford, J.C.; Lavin, D.P.; Adamson, S.R. In *Cell culture scale-up in stirred tank bioreactors*, Presented at the SIM Annual Meeting, Orlando, FL, 1990.

Gardiner, G.R.; Priston, R.; Stockdale, H. In *Studies on the production of baculoviruses in insect cell culture*. Proceeding of the first international colloquium on invertebrate pathology, Queen's University, Kingston, pp. 99-103, 1976.

Garnier, A.; Voyer, R.; Tom, R.; Perret, S.; Jardin, B.; Kamen, A. Dissolved carbon dioxide accumulation in a large scale and high density production of TGFβ receptor with baculovirus infected Sf-9 cells. *Cytotechnol.* **1996**, 22, 53-63.

Goss, S.; Singh, R.J.; Kalyanaraman, B. Bicarbonate enhances the peroxidase activity of Cu, Zn-superoxide dismutase. *J Biol. Chem.* **1999**, 274, 28233-28239.

Goudar, C.T.; Matanguihan, R.; Long, E.; Cruz, C.; Zhang, C.; Piret, J.M.; Konstantinov, K.B. Decreased pCO_2 accumulation by eliminating bicarbonate addition to high cell-density cultures. *Biotechnol. Bioeng.* **2006**, 96(6), 1107-1117.

Grace, T.D.C. Establishment of four strains of cells from insect tissues grown *in vitro*. *Nature* **1962**, 195, 788-789.

Granados, R. R.; Li, G.; Derkson, A. C. G.; McKenna, K. A. A new cell line from *Trichoplusia ni* (BTI-Tn-5B1-4) susceptible to *Trichoplusia ni* single enveloped nuclear polyhedrosis virus. *J. Invertebr. Pathol.,* **1994**, 64, 260-266.

Gray, D.R.; Chen, S.; Howarth, W.; Inlow, D.; Maiorella, B.L. CO_2 in large-scale and high-density CHO cell perfusion culture. *Cytotechnol.* **1996**, 22, 65-78.

Hawkins, C.L.; Morgan, P.E.; Davies, M.J. Quantification of protein modification by oxidants. *Free Radic. Biol. Med.* **2009**, 46, 965-988.

Hink, W.F. Established insect cell line from the cabbage looper,*Trichoplusia ni*. *Nature* **1970**, 226, 466–467.

Hollister, J.R.; Kawar, Z.S.; Jarvis, D.L. Engineering N-glycosylation pathways in the baculovirus-insect cell system. *Curr. Opin. Biotechnol.* **1998**, 9, 528-533.

Hollister, J.R.; Jarvis, D.L. Engineering lepidopteran insect cells for sialoglycoprotein production by genetic transformation with mammalian β1,4-galactosyltransferase and α2,6-sialyltransferase genes. *Glycobiology.* **2001**, 11(1), 1-9.

Ikonomou, L.; Bastin, G.; Schneider, Y.J.; Agathos, S.N. Design of an efficient medium for insect cell growth and recombinant protein production. *In vitro Cell. Dev. Bio. Animal* **2001**, 31, 549-559.

Ikonomou, L.; Schneider, Y.J.; Agathos, S.N. Insect cell culture for industrial production of recombinant proteins. *Appl. Microbiol. Biotechnol.* **2003**, 62, 1-20.

Ju, L.K.; Chase, G.G. Improved scale-up strategies of bioreactors. *Bioprocess Eng.* **1992**, 8, 49-53.

Kamen, A.A.; Tom, R.L.; Caron, A.W.; Chavarie, C.; Massie, B.; Archambautl, J. Culture of insect cells in a helical ribbon impeller bioreactor. *Biotechnol. Bioeng.* **1991**, 38, 619-628.

Kim, N.S.; Lee, G.M. Response of recombinant CHO cells to hyperosmotic pressure: Effect of Bcl-2 overexpression. *J. Biotechnol.* **2002**, 95, 237-248.

Kimura, R.; Miller, W.M. Effects of elevated pCO_2 and/or osmolality on the growth and recombinant tPA production of CHO cells. *Biotechnol. Bioeng.* **1996**, 52, 152-160.

Kimura, R.; Miller, W.M. Glycosylation of CHO-derived recombinant tPA produced under elevated pCO_2. *Biotechnol. Prog.* **1997**, 13, 311-317.

Krapf, R.; Berry, C.A.; Alpern, R.J.; Rector, F.C. Regulation of cell pH by ambient bicarbonate, carbon dioxide tension, and pH in the rabbit proximal convoluted tubule. *J. Clin. Invest.* **1988**, 81, 381-389.

Madshus, I.H. Regulation of intracellular pH in eukaryotic cells. *Biochem. J.* **1988**, 250, 1-8.

Maiorella, B.; Inlow, D.; Shauger, A.; Harano, D. Large-scale insect cell-culture for recombinant protein production. *Biotechnol.* **1988**, 6, 1406-1410.

Marks, D.M. Equipment design considerations for large scale cell culture. *Cytotechnol.* **2003**, 42, 21-33.

Matanguihan, R.; Sajan, E.; Konstantinov, K.; Zachariou, M.; Olson, C. Process and medium for mammalian cell culture under low dissolved carbon dioxide concentration. USA Patent 6338964, 2002.

Michaels, D.J.; Mallik, A.K.; Papoutsakis, E.T. Sparging and agitation-induced injury of cultured animal cells: Do cell-to-bubble interactions in the bulk liquid injure cells? *Biotechnol. Bioeng.* **1996**, 51, 399-409.

Mitchell-Logean, C.; Murhammer, D.W. Bioreactor headspace purging reduces dissolved carbon dioxide accumulation in insect cell cultures and enhances cell growth. *Biotechnol. Prog.* **1997**, 13, 875-877.

Mitsuhashi, J.; Maramorosch, K. Leafhopper Tissue Culture : Embryonic, Nymphal and Imaginal Tissues from Aseptic Insects. *Contrib. Boyce Thomson Inst*. **1964**, 22, 435-460.

Mostafa, S.S.; Gu, X. Strategies for improved dCO_2 removal in large-scale fed-batch culture. *Biotechnol. Prog.* **2003**, 19, 45-51.

Murhammer, D.W. Use of viral insecticides for pest control and production in cell culture. *Appl. Biochem. Biotechnol.* **1996**, 59, 199-219.

Murhammer, D.W.; Goochee, C.F. Sparged animal cell bioreactors: Mechanism of cell damage and Pluronic F-68 protection. *Biotechnol. Prog.* **1990**, 6, 391-397.

Murhammer, D.W.; Goochee, C.F. Scaleup of insect cell cultures: protective effects of Pluronic F-68. *Bio/Technol.* **1988**, 6, 1411-1418.

Onda, K.; Sada, E.; Kobayashi, T.; Kito, S.; Ito, K. Salting-out parameters of gas solubility in aqueous salt solutions. *J Chem Eng (Japan)* **1970**, 3, 18-24.

O'Reilly, D.R.; Miller, L.K.; Luckow, V.A. *Baculovirus Expression Vectors: a Laboratory Manual*; W. H. Freeman and Company: New York, 1992.

Oyaas, K.; Ellingsen, T.E.; Dyrset, N.; Levine, D.W. Hyperosmotic hybridoma cultures: Increased monoclonal antibody production with addition of glycine betaine. *Biotechnol. Bioeng.* **1994**, 44, 991-998.

Ozkan, P.; Mutharasan, R. A rapid method for measuring interacellular pH using BCECF-AM. *Biochim. et Biophy. Acta.* **2002**, 1572, 143-148.

Reddy, S.; Miller, W.M. Effects of abrupt and gradual osmotic stress on antibody production and content in hybridoma cells that differ in production kinetics. *Biotechnol. Prog.* **2004**, 10(2), 165-173.

Rhiel, M.; Mitchell-Logean, C.M.; Murhammer, D.W. Comparision of *Trichoplusia ni* BTI-Tn-5B1-4 (High Five^{TM}) and *Spodoptera frugiperda* Sf-9 insect cell line metabolism in suspension cultures. *Biotechnol. and Bioeng.* **1997**, 55(6), 909-920.

Ryu, J.S.; Lee, G.M. Effect of hyperosmotic stress on hybridoma cell growth and antibody production. *Biotechnol. Bioeng.* **1997**, 55, 565-570.

Schlaeger, E.J. Medium design for insect cell culture. *Cytotechnol.* **1996**, 20, 57-96.

Shoemaker, D.P.; Garland, C.W.; Steinfeld, J.I. *Experiments in Physical Chemistry*, 3rd ed.; McGraw-Hills book Company: New York, 1974.

Shuler, M.L.; Kargi, F. *Bioprocess Engineering – Basic Concepts*, 2nd ed.; Prentice-Hall, Inc.: New Jersey, 2006.

Sperandio, M.; Paul, E. Determination of carbon dioxide evolution rate during on-line gas analysis during dynamic biodegradation experiments. *Biotechnol. Bioeng.* **1997**, 53, 243-252.

Taticek, R.; Petersen, S.; Konstantinov, K.; Naveh, D. In *Effect of dissolved carbon dioxide and bicarbonate on mammalian cell metabolism and recombinant protein productivity in high density perfusion culture*. Presented at Cell Culture Engineering VI, San Diego, CA, 1998.

Vachon, V.; Paradis, M-J.; Marsolais, M.; Schwartz, JL.; Laprade, R. Endogenous K^+/H^+ exchange activity in the Sf-9 insect cell line. *Biochem.* **1995**, 34, 15157-15164.

Vaughn, J.L.; Goodwin, R.H.; Tompkins, G.J.; McCawley, P. The establishment of two cell lines from the insect *Spodoptera frugiperda lepidoptera noctuidae*. *In Vitro.* **1977**, 13, 213−217.

Vesela, A.; Wilhelm, J. The role of carbon dioxide in free radical reaction of the organism. *Physiol. Res.* **2002**, 51, 335-339.

Wang, Y.; Oberley, L.W.; Murhammer, D.W. Evidence of oxidative stress following the viral Infection of Two Lepidopteran Insect Cell Lines. *Free Radic. Biol. Med.* **2001**, 31(11), 148–155.

Weiss, S.A.; Smith, G.C.; Kalter, S.S.; Vaughn, J.L. Improved method for the production of insect cell cultures in large volume. *In Vitro* **1981**, 17, 495−5021.

Wyatt, S.S. Culture *in vitro* of tissue from Silkworm *Bombyx mori. J. Gen. Physiol.* **1956**, 39, 841−852.

Yang, J.D.; Gecik, P.; Collins, A.; Czarnecki, S.; Hsu, H.H.; Lasdun, A.; Sundaram, R.; Muthukumar, G.; Silberklang, M. Rational scale-up of a baculovirus-insect cell batch process based on medium nutritional depth. *Biotechnol. Bioeng.* **1996**, 52, 696-706.

Zhanghi, J.A.; Schmelzer, A.E.; Mendoza, T.P.; Knop, R.H.; Miller, W.M. Bicarbonate concentration and osmolality are key determinants in the inhibition of CHO cell polysialylation under elevated pCO_2 or pH. *Biotechnol. Bioeng.* **1999**, 65, 182-191.

Zhu, M.M.; Goyal, A.; Rank, D.L.; Gupta, S.K.; Vanden, B.T.; Lee, S.S. Effects of elevated pCO_2 and osmolality on growth of CHO cells and production of antibody-fusion protein B1: A case study. *Biotechnol. Prog.* **2005**, 21, 70-77.