Mechanism of CO2 inhibition in insect cell culture

Sucheta Gowthami Vajrala
University of Iowa

Copyright 2010 Sucheta Gowthami Vajrala

This dissertation is available at Iowa Research Online: http://ir.uiowa.edu/etd/611

Recommended Citation
http://ir.uiowa.edu/etd/611.

Follow this and additional works at: http://ir.uiowa.edu/etd

Part of the Chemical Engineering Commons
MECHANISM OF CO₂ 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
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

I am very grateful to my advisor, Prof. David Murhammer, for giving me the opportunity to work in his lab and for providing me the financial support throughout the course of this project. His guidance and patience were crucial for completion of this thesis. I would like to thank Dr. Tonya Peeples and Dr. Mani Subramanian for serving on my thesis committee. Thanks to Dr. Julie Jessop for her guidance in intracellular pH experiments. I also thank Prof. Mark Arnold for allowing me to use the glucose and lactate analyzer.

I appreciate Aaron Irons and Albert Kang for their assistance in bioreactor experiments. I acknowledge my lab mates Lopa Giri and Bhakti Bapat for their valuable help. I cannot thank my mother enough for her love and support throughout my life. I thank my father, my brother and my grandmother for their love and affection. I finally thank all other people who have rendered their help either directly or indirectly in completing this work in a successful manner.
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 CO₂ accumulation to inhibitory levels within the bioreactor. The current research investigated the effect of elevated CO₂ concentrations on insect cell growth and metabolism and the roles of oxidative stress and intracellular pH (pHi) in CO₂ 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°C and a pH of 6.2. The cells were exposed to a constant CO₂ concentration by purging the medium with CO₂ and the headspace with air. The experiments were repeated for different CO₂ 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 CO₂ concentration. Specifically, the PDT for 0-37, 73, 147, 183 and 220 mm Hg CO₂ 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 CO₂ 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
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** ........................................................................................................ viii

**LIST OF FIGURES** ...................................................................................................... ix

**NOMENCLATURE** ...................................................................................................... xi

**CHAPTER**

1. INTRODUCTION ........................................................................................................ 1
   1.1 Insect cell lines and baculovirus .......................................................... 1
   1.2 Cell metabolism ................................................................................. 2
   1.3 Medium formulation .......................................................................... 4
   1.4 Large-scale cell culture ........................................................................ 6
      1.4.1 Bioreactor operation ............................................................... 6
      1.4.2 Bioreactor types and scale-up ............................................. 7
   1.5 Mass transfer in stirred tank bioreactors ........................................... 8
      1.5.1 Agitation and hydrodynamic shear ..................................... 9
      1.5.2 Aeration and bubble damage ............................................. 10
      1.5.3 Major scale-up obstacle and CO₂ accumulation ................. 11
   1.6 Objective and hypothesis .................................................................. 13
   1.7 Overview ......................................................................................... 14

2. LITERATURE REVIEW .......................................................................................... 15
   2.1 Sources of CO₂ ................................................................................. 15
   2.2 Role of osmolality in CO₂ inhibition .............................................. 15
      2.2.1 Effect of CO₂ and/or osmolality on mammalian cells ........ 16
         2.2.1.1 Cell growth ......................................................... 17
         2.2.1.2 Cell death and apoptosis .................................. 20
         2.2.1.3 Cell metabolism ................................................ 21
         2.2.1.4 Protein production and glycosylation ............... 21
      2.2.2 Effect of CO₂ and/or osmolality on insect cells ................. 23
   2.3 Role of intracellular pH in CO₂ inhibition ...................................... 24
   2.4 Role of oxidative stress in CO₂ inhibition ...................................... 25
   2.5 CO₂ reduction strategy ................................................................. 25
   2.6 Current research contribution ......................................................... 27

3. MECHANISM OF CO₂ INHIBITION IN INSECT CELL CULTURE .... 29
   3.1 Introduction ..................................................................................... 29
   3.2 Materials and methods ................................................................. 34
      3.2.1 Cell line and medium ......................................................... 34
      3.2.2 Bioreactor ................................................................. 35
3.2.3 Analytical ................................................................. 38
3.2.4 Assays ................................................................. 39
  3.2.4.1 Lipid hydroperoxide assay ................................. 39
  3.2.4.2 Protein carbonyl assay ....................................... 40
  3.2.4.3 Intracellular pH ................................................. 41
3.3 Results and discussion ................................................. 42
  3.3.1 Effect of CO₂ on Sf-9 cell growth ......................... 42
    3.3.1.1 Population doubling time (PDT) ....................... 42
    3.3.1.2 Cell viability ..................................................... 45
  3.3.2 Effect of CO₂ on medium osmolality ....................... 45
  3.3.3 Effect of CO₂ on Sf-9 cell metabolism .................... 45
    3.3.3.1 Glucose consumption ....................................... 45
    3.3.3.2 Lactate production .......................................... 46
  3.3.4 Effect of CO₂ on oxidative stress ......................... 49
  3.3.5 Effect of CO₂ on intracellular pH (pHi) .................. 50
3.4 Conclusions ............................................................. 52

4. CONCLUSIONS AND FUTURE WORK .................................. 53

APPENDIX

A. GROWTH CURVES OF SF-9 CELLS AT DIFFERENT CO₂ CONCENTRATIONS ........................................... 54

REFERENCES ............................................................... 57
LIST OF TABLES

Table

1. Effect of different levels of CO₂ concentration and osmolality on CHO cell growth rates.................................................................19

2. Population Doubling Time (PDT) of Sf-9 cells at different CO₂ concentrations..........................................................................................44

3. Glucose production and lactate consumption rates of Sf-9 cells at different CO₂ concentrations .................................................................48
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Major metabolic components consumed and produced by Sf-9 and Tn-5 cells during normal growth conditions.</td>
</tr>
<tr>
<td>2.</td>
<td>Schematic of CO₂ and ion transfer between gas-liquid interface and cell membrane.</td>
</tr>
<tr>
<td>4.</td>
<td>Experimental set-up.</td>
</tr>
<tr>
<td>5.</td>
<td>Typical growth curves of Sf-9 cells at different CO₂ concentrations.</td>
</tr>
<tr>
<td>6.</td>
<td>Population Doubling Time (PDT) of Sf-9 cells at different CO₂ concentrations.</td>
</tr>
<tr>
<td>7.</td>
<td>Glucose concentration of Sf-9 cell culture in shaker flask and in bioreactor controlled at different CO₂ concentrations.</td>
</tr>
<tr>
<td>8.</td>
<td>Lactate concentration of Sf-9 cell culture in shaker flask and in bioreactor controlled at different CO₂ concentrations.</td>
</tr>
<tr>
<td>9.</td>
<td>Specific lactate production rate of Sf-9 cells at different CO₂ concentrations.</td>
</tr>
<tr>
<td>10.</td>
<td>Lipid hydroperoxide concentrations of Sf-9 cells grown in shaker flask and in bioreactor controlled at different CO₂ concentrations.</td>
</tr>
<tr>
<td>11.</td>
<td>Protein carbonyl concentrations of Sf-9 cells grown in shaker flask and in bioreactor controlled at different CO₂ concentrations.</td>
</tr>
<tr>
<td>12.</td>
<td>Typical excitation scans (410 to 515 nm) of uninfected Sf-9 cells and Sf-9 cells during calibration.</td>
</tr>
<tr>
<td>A1</td>
<td>Growth curves of Sf-9 cells exposed to 0-37 mm Hg CO₂.</td>
</tr>
<tr>
<td>A2</td>
<td>Growth curves of Sf-9 cells exposed to 73 mm Hg CO₂.</td>
</tr>
<tr>
<td>A3</td>
<td>Growth curves of Sf-9 cells exposed to 147 mm Hg CO₂.</td>
</tr>
<tr>
<td>A4</td>
<td>Growth curves of Sf-9 cells exposed to 183 mm Hg CO₂.</td>
</tr>
</tbody>
</table>
A5 Growth curves of Sf-9 cells exposed to 220 mm Hg CO₂
# LIST OF NOMENCLATURE

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcMNPV</td>
<td><em>Autographa californica</em> multiple nucleopolyhedrovirus</td>
</tr>
<tr>
<td>APOX</td>
<td>Ascorbate peroxidase</td>
</tr>
<tr>
<td>BEVS</td>
<td>Baculovirus Expression Vector System</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney cells</td>
</tr>
<tr>
<td>C*-C</td>
<td>Concentration difference across the gas-liquid interface</td>
</tr>
<tr>
<td>[CO₂]⁺</td>
<td>CO₂ aqueous concentration in equilibrium with the gas phase</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>Copper zinc superoxide dismutase</td>
</tr>
<tr>
<td>Dᵢ</td>
<td>Impeller diameter</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved oxygen</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>H</td>
<td>Henry’s law constant</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>N</td>
<td>Agitation speed</td>
</tr>
<tr>
<td>Nₚ</td>
<td>Power number</td>
</tr>
<tr>
<td>OTR</td>
<td>Oxygen transfer rate</td>
</tr>
<tr>
<td>OUR</td>
<td>Oxygen uptake rate</td>
</tr>
<tr>
<td>P</td>
<td>Power</td>
</tr>
<tr>
<td>P_{CO₂}</td>
<td>Partial pressure of CO₂</td>
</tr>
<tr>
<td>PDT</td>
<td>Population doubling time</td>
</tr>
<tr>
<td>Re</td>
<td>Reynolds number</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RQ</td>
<td>Respiratory Quotient</td>
</tr>
<tr>
<td>Sf-9</td>
<td><em>Spodoptera frugiperda</em> cell line</td>
</tr>
<tr>
<td>Sf-21</td>
<td><em>Spodoptera frugiperda</em> IPLB-Sf21-AE cell line</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>T&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Temperature</td>
</tr>
<tr>
<td>Tn-5</td>
<td><em>Trichoplusia ni</em> BTI-Tn-5B1-4 cell line</td>
</tr>
<tr>
<td>V</td>
<td>Volume</td>
</tr>
<tr>
<td>k&lt;sub&gt;L&lt;/sub&gt;a</td>
<td>Mass transfer coefficient</td>
</tr>
<tr>
<td>pH&lt;sub&gt;e&lt;/sub&gt;</td>
<td>Extracellular pH</td>
</tr>
<tr>
<td>pH&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Intracellular pH</td>
</tr>
<tr>
<td>q&lt;sub&gt;AB&lt;/sub&gt;</td>
<td>Specific antibody production rate</td>
</tr>
<tr>
<td>q&lt;sub&gt;amm&lt;/sub&gt;</td>
<td>Specific ammonia production rate</td>
</tr>
<tr>
<td>q&lt;sub&gt;glu&lt;/sub&gt;n</td>
<td>Specific glutamine consumption rate</td>
</tr>
<tr>
<td>q&lt;sub&gt;glu&lt;/sub&gt;c</td>
<td>Specific glucose consumption rate</td>
</tr>
<tr>
<td>q&lt;sub&gt;lac&lt;/sub&gt;</td>
<td>Specific lactate production rate</td>
</tr>
<tr>
<td>rwv</td>
<td>Rotating wall vessel</td>
</tr>
<tr>
<td>vvm</td>
<td>Volume of gas flow per volume of liquid per minute</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactisodase</td>
</tr>
<tr>
<td>ρ</td>
<td>Density</td>
</tr>
<tr>
<td>μ</td>
<td>Viscosity</td>
</tr>
</tbody>
</table>
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 $2.4 \times 10^{17}$ mol/cell-s and 2.8 to $7.4 \times 10^{17}$ 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 (Rhie 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 (Rhie et al., 1997).

![Figure 1: Major metabolic components consumed and produced by Sf-9 and Tn-5 cells during normal growth conditions](image)

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 $5 \times 10^{-17}$ mol/cell-s and 5 to $16 \times 10^{-17}$ 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 $8.6 \times 10^{-17}$ mol/cell-s and the oxygen consumption rate of 5.5 to $8.06 \times 10^{-17}$ 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 yeastolat-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
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

\[ P = \frac{N \cdot D^3}{D} \]

where

- \( P \) is the power per volume
- \( N \) is the impeller speed
- \( D \) is the impeller diameter
\[
P/V = N_p N^3 D_i^5 \rho / V, \tag{1.1}
\]
where \(N_p\) = impeller power number, \(N\) = agitation speed, \(D_i\) = impeller diameter, \(\rho\) = 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 \((k_{L,a})\) 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, \(k_{L,a}\) and the driving concentration difference \((C^*-C)\) across the gas-liquid interfacial area \((OTR = k_{L,a} (C^*-C))\). 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,
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 CO₂, 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 CO₂ 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 CO₂ 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 CO₂ accumulation within the bioreactor.
CO₂ 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 CO₂ 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 CO₂ 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 CO₂ and its dissociation into HCO₃⁻ and H⁺ ions, in cell culture medium and in the cell cytoplasm, may induce oxidative stress, decrease intracellular pH (pHᵢ) and alter medium osmolality. The role of CO₂ accumulation in oxidative stress is primarily due to the formation of carbonate anion (CO₃⁻) and peroxymonocarbonate anion (HCO₄⁻) (Goss et al., 1999; Vesela and Wilhelm, 2002). The formation of CO₃⁻ and HCO₄⁻ occurs mainly in the presence of strong oxidizing agents like H₂O₂. The viral infection of cells produces significant levels of H₂O₂ and hence would be expected to lead to the production of CO₃⁻ and HCO₄⁻ in the presence of HCO₃⁻. CO₃⁻ and HCO₄⁻ are strong oxidizing components and can damage proteins and lipids, thereby altering cell metabolism. The formation of CO₃⁻ and HCO₄⁻ occurs due to the following processes:

1. Production of carbonate anion (CO₃⁻)
   a. H₂O₂ can be converted to hydroxyl radical (OH⁻) by the following mechanism:
i. \( \text{SOD} - \text{Cu}^{2+} + \text{H}_2\text{O}_2 \leftrightarrow \text{SOD} - \text{Cu}^{1+} + \text{O}_2^- + 2\text{H}^+ \) \hspace{1cm} (1.2)

ii. \( \text{SOD} - \text{Cu}^{1+} + \text{H}_2\text{O}_2 \leftrightarrow \text{SOD} - \text{Cu}^{2+} - \text{OH}^- + \text{OH}^- \) \hspace{1cm} (1.3)

b. The resulting \( \text{OH}^- \) oxidizes \( \text{HCO}_3^- \) to produce \( \text{CO}_3^- \):

i. \( \text{SOD} - \text{Cu}^{2+} - \text{OH}^- + \text{HCO}_3^- \leftrightarrow \text{SOD} - \text{Cu}^{2+} + \text{H}_2\text{O} + \text{CO}_3^- \) \hspace{1cm} (1.4)

2. Production of peroxymonocarbonate anion (\( \text{HCO}_4^- \))

a. \( \text{H}_2\text{O}_2 \) Reacts with \( \text{HCO}_3^- \) to produce \( \text{HCO}_4^- \)

i. \( \text{H}_2\text{O}_2 + \text{HCO}_3^- \leftrightarrow \text{H}_2\text{O} + \text{HCO}_4^- \) \hspace{1cm} (1.5)

The increase in \( \text{CO}_2 \) concentration leads to intracellular acidification in mammalian cells due to the accumulation of higher levels of \( \text{H}^+ \) ions in the cell cytoplasm (deZengotita et al., 2002; Taticek et al., 1998 and Adler et al., 1965). The decreased \( \text{pH}_i \) (increase in \( \text{H}^+ \) ions) has detrimental effects on cellular metabolism by interfering with the enzyme activity. The contribution of \( \text{CO}_2 \) accumulation to medium osmolality is largely due to the maintenance of cell culture at constant \( \text{pH} \), i.e., through the addition of base. In CHO cell cultures the combination of \( \text{CO}_2 \) 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 \( \text{CO}_2 \) accumulation are expected to have minimal/no effect on insect cell cultures.

1.6 Objective and Hypothesis:

This research investigated the effect of specific \( \text{CO}_2 \) concentrations on insect cell growth and metabolism. A well-controlled bioreactor maintained at a constant \( \text{CO}_2 \) concentration was used to determine the inhibitory effect of \( \text{CO}_2 \) on uninfected Sf-9 cell
growth and metabolism. Also, the mechanism of the inhibitory effect of CO\textsubscript{2} was studied by evaluating the role of oxidative stress. Furthermore, experiments leading to the determination of the effect of elevated CO\textsubscript{2} on pH\textsubscript{i} in insect cells were conducted.

The hypothesis of this research work is that the CO\textsubscript{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\textsubscript{2} concentration on uninfected insect cell growth and metabolism and the role of oxidative stress and pH\textsubscript{i} in CO\textsubscript{2} inhibition. Chapter 2 explains the previous studies conducted on the CO\textsubscript{2} inhibition in insect and mammalian cells and several different techniques suggested to overcome CO\textsubscript{2} inhibitory affect. Chapter 3 discusses the current research work conducted on the effect and mechanism of CO\textsubscript{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 CO₂ 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 CO₂ accumulation on growth and productivity in different cell lines, role of osmolality, oxidative stress and intracellular pH in CO₂ inhibition, and also approaches that can be used to reduce CO₂ accumulation, and/or reduce the adverse effects thereof.

2.1 Sources of CO₂

The physiological concentration of CO₂ 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 CO₂ partial pressure of 30-50 mm Hg). The accumulation of higher CO₂ concentrations in bioreactors is primarily due to the addition of CO₂ from biotic sources, e.g., cellular respiration, and the addition of CO₂ from abiotic sources, e.g., addition of bicarbonate as a buffer to the cell culture medium and addition of CO₂ or NaHCO₃ to control the pH of the cell culture medium (Goudar et al., 2006).

2.2 Role of Osmolality in CO₂ Inhibition

As CO₂ accumulates, it can easily transfer between the gas-liquid interface and through the cell membrane (Figure 3) and dissociate into bicarbonate (HCO₃⁻) and hydronium ions (H⁺) in aqueous solutions. The HCO₃⁻ 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.

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 x 10\(^6\) to 5.9 x 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 CO₂ 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 CO₂ 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 CO₂ 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

<table>
<thead>
<tr>
<th>Experimental values CO$_2$ concentration (mm Hg) / osmolality (mOsm/kg)</th>
<th>Control values CO$_2$ concentration (mm Hg) / osmolality (mOsm/kg)</th>
<th>Effect on growth rate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated CO$_2$ concentration and lower osmolality levels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140 mm Hg / 310 mOsm/kg</td>
<td>36 mm Hg / 310 mOsm/kg</td>
<td>negligible change</td>
<td></td>
</tr>
<tr>
<td>195 mm Hg / 310 mOsm/kg</td>
<td>36 mm Hg / 310 mOsm/kg</td>
<td>negligible change</td>
<td></td>
</tr>
<tr>
<td>250 mm Hg / 310 mOsm/kg</td>
<td>36 mm Hg / 310 mOsm/kg</td>
<td>30% decrease</td>
<td></td>
</tr>
<tr>
<td>Lower CO$_2$ concentration and elevated osmolality levels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36 mm Hg / 342 mOsm/kg</td>
<td>36 mm Hg / 310 mOsm/kg</td>
<td>negligible change</td>
<td></td>
</tr>
<tr>
<td>36 mm Hg / 357 mOsm/kg</td>
<td>36 mm Hg / 310 mOsm/kg</td>
<td>negligible change</td>
<td></td>
</tr>
<tr>
<td>36 mm Hg / 376 mOsm/kg</td>
<td>36 mm Hg / 310 mOsm/kg</td>
<td>negligible change</td>
<td></td>
</tr>
<tr>
<td>36-70 mm Hg / 445 mOsm/kg</td>
<td>36-70 mm Hg / 316 mOsm/kg</td>
<td>60% decrease</td>
<td></td>
</tr>
<tr>
<td>Elevated CO$_2$ concentration and elevated osmolality levels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140 mm Hg / 337 mOsm/kg</td>
<td>36 mm Hg / 310 mOsm/kg</td>
<td>22% decrease</td>
<td></td>
</tr>
<tr>
<td>140 mm Hg / 370 mOsm/kg</td>
<td>36 mm Hg / 310 mOsm/kg</td>
<td>35% decrease</td>
<td></td>
</tr>
<tr>
<td>195 mm Hg / 361 mOsm/kg</td>
<td>36 mm Hg / 310 mOsm/kg</td>
<td>45% decrease</td>
<td></td>
</tr>
<tr>
<td>195 mm Hg / 415 mOsm/kg</td>
<td>36 mm Hg / 310 mOsm/kg</td>
<td>63% decrease</td>
<td></td>
</tr>
<tr>
<td>250 mm Hg / 376 mOsm/kg</td>
<td>36 mm Hg / 310 mOsm/kg</td>
<td>45% decrease</td>
<td></td>
</tr>
<tr>
<td>168 mm Hg / 375 mOsm/kg</td>
<td>90 mm Hg / 325 mOsm/kg</td>
<td>35% decrease</td>
<td></td>
</tr>
</tbody>
</table>
2.2.1.2 Cell Death and Apoptosis

Various contradictory propositions were made regarding the effect of CO\textsubscript{2} and osmolality on cell viability. Experiments conducted in a 10 L bioreactor using CHO cells demonstrated that increasing the CO\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{2} concentration on apoptotic cell death was minimal at a constant osmolality. Furthermore, increasing CO\textsubscript{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\% (Zhang \textit{et al.}, 1999).

\subsection*{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 \textit{et al.}, 1996). Also, the effect of elevated CO\(_2\) concentration on the production of \(\beta\)-gal and TGF\(\beta\)-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 \(\beta\)-gal and TGF\(\beta\) 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 \textit{Trichoplusia ni} Tn-368 cell growth (Gardiner \textit{et al.}, 1976). Increasing the medium osmolality to 510 mOsm/kg with the addition of 30 mM NaCl to EX-CELL 405 serum-free medium had minimal effect on cell growth and a slight inhibitory effect on recombinant protein synthesis in the Tn-5 cell line (Yang \textit{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 CO₂ Inhibition

The activity of several cellular metabolic enzymes, e.g., phosphofructokinase, are pH sensitive. Changes in intracellular pH (pHᵢ) affects cell metabolism, ion conductivities, protein synthesis and cell cycle (Madshus et al., 1988). The intracellular dissociation of CO₂ results in the accumulation of H⁺ ions in the cell cytoplasm leading to reduce intracellular pH (pHᵢ) even when the extracellular pH (pHₑ) is controlled. Although Na⁺/H⁺, Cl⁻/HCO₃⁻ and similar antiporters counteract the intracellular acidification effect in mammalian cells (Madshus et al., 1988), the pHᵢ 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 CO₂ concentration decreases pHᵢ (deZengotita et al., 2002; Tatricek et al., 1998; Adler et al., 1965). Studies conducted on BHK-21 cells maintained at pHₑ 6.8 in perfusion culture showed that increase in CO₂ concentration from 35 mm Hg to 270 mm Hg decreased the pHᵢ from 7.2 to 6.9 (Tatricek et al., 1998). In contrast, experiments conducted on hybridoma cells in T-flasks demonstrated that increased CO₂ concentration (up to 140 mm Hg) decreased pHᵢ by 0.1 – 0.2 units and that pHᵢ did not decrease further with increased CO₂ concentration above 140 mm Hg.

The pHᵢ 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 pHᵢ of Sf-9 cells is largely dependant on the medium composition especially the extracellular concentration of K⁺. However, previous
investigations conducted demonstrated that the pHi 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 CO₂ Inhibition

Reactive Oxygen Species (ROS), e.g., superoxide radical (O₂⁻), hydroxyl radical (·OH), and hydrogen peroxide (H₂O₂), 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 O₂⁻ to H₂O₂) and ascorbate peroxidase (APOX that reduces H₂O₂ to H₂O) that are present in both Sf-9 and Tn-5 cells (Wang et al., 2001). Additionally, Tn-5 cells contain catalase (CAT) for reducing H₂O₂ to H₂O. 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 CO₂ accumulation, combine with H₂O₂ (produced as a result of the viral infection of insect cells) to form strong oxidizing components, e.g., CO₃⁻ and HCO₄⁻, thus inducing oxidative stress (eq. 1.2 – 1.5). Hence, it was hypothesized that CO₂ accumulation induces oxidative stress only in infected insect cells.

2.5 CO₂ Reduction Strategy

The dissolved CO₂ concentration in high cell density CHO and BHK cell cultures can be significantly reduced by eliminating the CO₂ addition from abiotic sources (Matanguihan et al., 2002; Goudar et al., 2006). The addition of CO₂ or NaHCO₃ to
control pH can be replaced with the addition of Na₂CO₃. 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 NaHCO₃ (used for pH control) with Na₂CO₃ reduced the dissolved CO₂ 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 CO₂ can be achieved by increasing the bubble size, sparge flow rates, and impeller position (Mostafa and Gu, 2003; Gray et al., 1996). Dissolved CO₂ 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 CO₂ removal (Gray et al., 1996). Similarly, in a CHO cell fed-batch culture, CO₂ was removed by macro sparging (Mostafa and Gu, 2003).

Dissolved CO₂ 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 CO₂ concentrations from 600 to ~75 mm Hg (based on a CO₂ 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 \( \sim 5 \times 10^6 \) to \( \sim 8 \times 10^6 \) and from \( \sim 3 \times 10^6 \) to \( \sim 8 \times 10^6 \) 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\textsubscript{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 (~ 20 x 10\textsuperscript{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\textsubscript{2} to inhibitory levels within the bioreactor. Cellular metabolism is the primary source of CO\textsubscript{2} accumulation, whereas the bicarbonate (added as a buffer) addition is a secondary source. The respiratory quotient, i.e., the molar ratio of CO\textsubscript{2} produced to O\textsubscript{2} consumed which is typically ~1 for Sf-9 cells, can be used to estimate the cellular CO\textsubscript{2} production rate if the dissolved O\textsubscript{2} rate is known (Kamen \textit{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, \textit{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, \textit{et al.}, 1990; Marks, 2003). These small bubbles often dissolve in the cell culture medium before reaching the surface without stripping CO\textsubscript{2}, thereby leaving surface mass transfer as the only means of CO\textsubscript{2} removal. Also, in large-scale bioreactors the surface to volume ratio decreases with increasing bioreactor size, thereby further reducing dissolved CO\textsubscript{2} removal to exacerbate CO\textsubscript{2} accumulation within the bioreactor.
As CO₂ accumulates in the bioreactor, it dissolves in the cell culture medium as well as in the cell cytoplasm (i.e., CO₂ passes freely across cell membranes) and dissociates into HCO₃⁻ and H⁺ ions (Equation 3.1).

\[
\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^- \tag{3.1}
\]

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).

\[
P_{\text{CO}_2} = H \times [\text{CO}_2]^* \tag{3.2}
\]

Where \([\text{CO}_2]^*\) is the CO₂ concentration in equilibrium with the gas phase, \(P_{\text{CO}_2}\) is the partial pressure of CO₂ and \(H\) is the Henry’s law constant (Pa/M). The Henry’s law constant for CO₂ dissolved in pure water at 27°C (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).

\[
H = 101,330 \times \exp[11.549 - (2440.4/T_L)] \tag{3.3}
\]

Where \(T_L\) is the water temperature (K). The final value for the solubility of CO₂ 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).

HCO₃⁻ and the base (e.g., KOH) added to control extracellular pH (pHₑ) 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 CO₂ concentration that is typically required for normal cell growth is 30-50 mm Hg, whereas CO₂ 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 CO₂ 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 CO₂ concentration from 36 to 145 mm Hg (osmolality – 310 to 340 mOsm/kg) decreased the maximum cell density (from 8.9 x 10⁶ to 5.9 x 10⁶ cell/mL), viability (from 79% to 58%) and the final productivity (by 69%) (Gray et al., 1996). Similarly, an increase in CO₂ concentration from 53 to 167 mm Hg decreased the CHO cell growth by 41% (Drapeau et al., 1990), whereas an increase in CO₂ 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 CO₂ 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 CO₂ and osmolality (Table 1). However, conflicting arguments have been made on the combined effect of CO₂ 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 CO₂ 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 $\beta$-gal and TGF$\beta$ 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 HCO₃⁻ (produced from the dissociation of CO₂) with H₂O₂ (produced from the viral infection of cells), to produce carbonate anion (CO₃²⁻) and peroxymonocarbonate anion (HCO₄⁻) (eq. 1.2 – 1.5). CO₃²⁻ and HCO₄⁻ 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 H₂O₂ occurs only in the presence of viral infection, it is hypothesized that CO₂ accumulation only enhances oxidative stress in infected cells.

Several different techniques have been investigated to overcome CO₂ accumulation, including (1) the replacement of bicarbonate buffer with MOPS-histidine buffer, and NaHCO₃ with Na₂CO₃ 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 CO₂ concentrations (Croughan, 2009).

In a 1.5 L bioreactor, purging the headspace with air reduced the dissolved CO₂ 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 10⁶ and from ~3-5 to ~8-10 x 10⁶ cells/mL in Sf-9 and Tn-5 cells, respectively. However, the CO₂ measurements taken may not have been precise since some CO₂ may have volatilized during the transfer of sample from the bioreactor for offline measurement.
The current research work studied the effect of specific CO₂ concentrations on uninfected insect cell growth and metabolism. Also, the mechanism of CO₂ 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 CO₂ on pHᵢ in insect cells was also discussed. Some of the limitations in previous research work, such as offline measurement of CO₂ using a blood gas analyzer or by estimating the CO₂ concentration based on mass transfer equations, have been overcome by conducting all experiments in a well-controlled bioreactor equipped with an autoclavable CO₂ 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 cm² 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 10⁶ cells/mL. An orbital shaker (Thermo Scientific, Waltham, MA), maintained at a temperature of 27°C 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 CO₂ accumulation within the bioreactor, the headspace was continuously purged with air at a flow rate of 200 mL/min. The CO₂ concentration in the cell culture medium was monitored on-line using YSI 8505 autoclavable CO₂ probe with YSI 8500 CO₂ monitor (Yellow Springs Instruments, Yellow Springs, OH). The CO₂ 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\textsubscript{2} probes
The bioreactor was seeded with Sf-9 cells at a density of $0.8 \times 10^6$ 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\textsubscript{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 –20\textdegree C 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 –80\textdegree C freezer until assayed. Prior to assaying, the samples were removed from the –80\textdegree C 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 4\degree C 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 (\( \mu \)) of Sf-9 cells grown at a given CO\textsubscript{2} concentration was obtained from the slope of the logarithm of viable cell density in exponential growth phase region versus time. This \( \mu \) was used to find the Population Doubling Times (PDT) of Sf-9 cells cultivated at a given CO\textsubscript{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 H₂O (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⁻¹cm⁻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 x 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

3.3.1 Effect of CO$_2$ on SF-9 Cell Growth

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 CO₂ concentrations. The increase in CO₂ 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 CO₂ 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 different CO₂ concentrations
Figure 6: Population Doubling Time (PDT)* of Sf-9 cells at different CO₂ concentrations

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

Table 2: Population Doubling Time of Sf-9 cells at different CO₂ concentrations

<table>
<thead>
<tr>
<th>CO₂ (mm Hg)</th>
<th>PDT for several repetitions (h)</th>
<th>Mean</th>
<th>n</th>
<th>PDT* (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-37</td>
<td>26.2  20.9  22.6</td>
<td>-</td>
<td>-</td>
<td>23.2</td>
</tr>
<tr>
<td>73</td>
<td>29.9  35.6  31.8</td>
<td>-</td>
<td>-</td>
<td>32.4</td>
</tr>
<tr>
<td>147</td>
<td>36.3  50.2  34.8</td>
<td>31.1</td>
<td>-</td>
<td>38.1</td>
</tr>
<tr>
<td>183</td>
<td>43.6  40.5  44.7</td>
<td>-</td>
<td>-</td>
<td>43.0</td>
</tr>
<tr>
<td>220</td>
<td>102b  51.0  58.7  65.4</td>
<td>-</td>
<td>-</td>
<td>69.3</td>
</tr>
<tr>
<td>Shaker</td>
<td>26.0  27.6  26.8  20.9  29.5  21.8  21.8</td>
<td>24.9</td>
<td>7</td>
<td>24.9</td>
</tr>
</tbody>
</table>

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 ($2.6 \times 10^{-17}$ 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 x 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 x 10$^{-17}$ mol/cell-s.

![Glucose concentration of Sf-9 cell culture in shaker flask and in bioreactor controlled at different CO$_2$ concentrations](image)

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 x 10$^{-17}$ and 2.6 x 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 CO₂ concentration (Figure 8). Cells grown in shaker flasks and cells grown in the bioreactor controlled at 0–37 mm Hg CO₂ 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 $-3.0 \times 10^{-19}$ to $10.2 \times 10^{-19}$ mol/cell-s with the increase in CO₂ concentration from 0-37 to 220 mm Hg (Figure 9). These results demonstrated that the exposure of Sf-9 cells to CO₂ 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 CO₂ concentrations](image)

* 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

<table>
<thead>
<tr>
<th>CO$_2$ (mm Hg)</th>
<th>No. of experiments (n)</th>
<th>Glucose conc. (10$^{-17}$ mol/cell-s)</th>
<th>Lactate conc. (10$^{-19}$ mol/cell-s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-37</td>
<td>1</td>
<td>-3.2$^a$</td>
<td>-3.0</td>
</tr>
<tr>
<td>73</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>147</td>
<td>1</td>
<td>-2.9</td>
<td>1.6</td>
</tr>
<tr>
<td>183</td>
<td>1</td>
<td>-2.5</td>
<td>5.4</td>
</tr>
<tr>
<td>220</td>
<td>2</td>
<td>-2.9 ± 0.1$^b$</td>
<td>10.2 ± 1.5$^b$</td>
</tr>
<tr>
<td>Shaker flask</td>
<td>3</td>
<td>-2.6 ± 1.6$^c$</td>
<td>-2.4 ± 1.0$^c$</td>
</tr>
</tbody>
</table>

a-negative sign indicates substrate consumption; b-range; c-95% confidence levels
3.3.4 Effect of CO\textsubscript{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\textsubscript{2} concentrations was measured to determine the role of oxidative stress in CO\textsubscript{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\textsubscript{2} concentrations (Figure 10). Similarly, the results of protein carbonyl assay indicated that elevated CO\textsubscript{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\textsubscript{2} concentrations](image)

* range; ** 95% confidence levels
Figure 11: Protein carbonyl concentrations of Sf-9 cells growth in shaker flask and in bioreactor controlled at different CO\textsubscript{2} concentrations

\* range

3.3.5 Effect of CO\textsubscript{2} on Intracellular pH (pH\textsubscript{i})

Experiments were conducted to develop the calibration curve and to determine the pH\textsubscript{i} of Sf-9 cells. An independent calibration curve was developed each time prior to the measurement of the pH\textsubscript{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\textsubscript{i} caused due to elevated CO\textsubscript{2} concentrations has to be conducted. Such experiments should be conducted with extreme caution to prevent the escape of CO\textsubscript{2} from the sample culture medium while performing the pH\textsubscript{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
3.4 Conclusions

The increase in CO₂ concentration resulted in reduced cell growth and increased PDT in uninfected Sf-9 cells. Elevated CO₂ concentration did not significantly alter cell viability, medium osmolality and the specific glucose consumption rate. However, increased CO₂ 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 CO₂ inhibition in uninfected Sf-9 cells. Future work related to the role of pHᵢ in CO₂ inhibition in uninfected Sf-9 cells will yield useful information in finding the mechanism behind CO₂ inhibition.

Figure 13: Typical calibration curve for pHᵢ
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

GROWTH CURVES OF SF-9 CELLS AT DIFFERENT CO₂ CONCENTRATIONS

Figure A1: Growth curves of SF-9 cells exposed to 0-37 mm Hg CO₂

Figure A2: Growth curves of SF-9 cells exposed to 73 mm Hg CO₂
Figure A3: Growth curves of Sf-9 cells exposed to 147 mm Hg CO₂

Figure A4: Growth curves of Sf-9 cells exposed to 183 mm Hg CO₂
Figure A5: Growth curves of Sf-9 cells exposed to 220 mm Hg CO₂
REFERENCES


