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THE UNIVERSITY OF ALBERTA

THE ROLE OF OXYGEN LEVELS AND FLUCTUATIONS IN ANTIBIOTIC FERMENTATIONS :

SCALE–DOWN AND CONTROL STUDIES

By

P.K. YEGNESWARAN 

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMICAL ENGINEERING

EDMONTON, ALBERTA

FALL 1990

ISBN 0-315-65039-7

THE UNIVERSITY OF ALBERTA
RELEASE FORM

NAME OF AUTHOR: P.K. Yegneswaran

TITLE OF THESIS: The Role of Oxygen Levels and Fluctuations in Antibiotic Fermentations : Scale–down and Control Studies

DEGREE: Doctor of Philosophy

YEAR THIS DEGREE GRANTED: 1990

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

The Role of Oxygen Levels and Fluctuations in Antibiotic Fermentations : Scale–down and Control Studies

submitted by P.K. Yegneswaran in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dedication

This thesis is dedicated to my parents.

ABSTRACT

Cells grown in production–scale stirred bioreactors experience fluctuating concentrations due to local variations in supply and consumption of oxygen and nutrients. A novel experimental technique for studying the effects of fluctuations in local conditions on growth and productivity of cells was developed, using a Monte Carlo approach, to simulate the distribution of circulation times in large fermenters. The method was demonstrated by carrying out experiments to simulate fluctuations in dissolved oxygen concentrations, and evaluate its effect on growth and ethanol production in batch and fed–batch cultures of *Saccharomyces cerevisiae*. A small fermenter was driven through cycles of aeration, with the period of each cycle controlled by a Monte Carlo method to give a log–normal distribution of circulation times typical of large fermenters. The Monte Carlo air cycling method was compared with continuous aeration and fixed–period cycling. Reproducible differences in growth and ethanol accumulation during different modes of aeration showed that the distribution of circulation times is an important factor to be considered during fermentor scale–up.

The key biosynthetic enzymes responsible for production of β–lactam antibiotics, penicillin N and cephamycin C by *Streptomyces clavuligerus* are affected by dissolved oxygen (DO) levels. Experiments to simulate large–scale DO fluctuations using the Monte Carlo method were carried out in batch cultures of *Streptomyces clavuligerus* to evaluate the effects on growth and antibiotic production. Lower levels of total antibiotics and cephamycin C were obtained in the Monte Carlo and periodic aeration experiments due to lower average dissolved oxygen levels compared to the continuous aeration experiments. The final cephamycin C levels were lower for the Monte Carlo aeration experiments compared to the periodic case, consistent with lower expression of the biosynthetic enzymes.

A dissolved oxygen control system, with constant shear and mass transfer

conditions, was used to study the effect of different constant DO levels and stepwise change in DO levels on growth and antibiotic production in *S. clavuligerus*. A 2.4 fold increase in final cephamycin C yield was obtained by maintaining DO at 100 % saturation during the growth phase. No benefit was observed in controlling DO levels once growth had ceased.

Carbon dioxide evolution rate is an important on–line measurement for the estimation of the metabolic state of the culture as in determining the cessation of growth for DO control. Fluctuations in pH and head–space pressure in a fermentor introduce temporary changes in off–gas carbon dioxide concentrations. These changes were quantified using a simple model based on kinetics of carbon dioxide hydration and gas–liquid mass transfer. The model was verified experimentally.

ACKNOWLEDGEMENTS

I would like to thank those people who made the work in this thesis an enjoyable experience.

Dr. Murray Gray, for all the guidance, and constant encouragement.

Dr. Brad Thompson, for all the help and use of facilities at the Alberta Research Council.

Dr. Dennis Ward, for help with the experimental equipment and valuable suggestions.

Dave, and Namdev for their useful suggestions.

NSERC and University of Alberta for the funding for this project.

Twyla Malcolm for assistance with experiments

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NOMENCLATURE

$[CO_2]$	dissolved CO_2 concentration (M)
D	dilution rate (h^{-1})
DCW	dry cell weight (g/L)
DO	dissolved oxygen (% saturation)
e(k)	error between the setpoint and the filtered DO measuremet (% saturation)
H	Henry's constant for CO_2 (M atm^{-1})
$[HCO_3^-]$	bicarbonate concentration (M)
k_la	mass transfer coefficient for O_2, CO_2 (h^{-1})
K_C	proportional constant for PI controller
K_I	integral constant for PI controller
OUR	oxygen uptake rate (mmoles/L/h)
P^o	reference pressure (1 atm)
pO_2	dissolved oxygen (% saturation)
pCO_2	dissolved carbon dioxide (M)
q	specific rate of production or consumption (g or mg/g DCW/h)
Q	gas flow rate (standard litres per minute)
R_n	normally distributed random number
R_u	uniformly distributed random number
t_c	circulation time (s)
$\bar{t}_c$	mean circulation time (s)
t_p	pulse time during experimental cycle (s)
u(k)	control signal (litres per minute of oxygen)
$\Delta u(k)$	incremental control signal (litres per minute of oxygen)
V_l	liquid volume (litres)

x_i, x_o	mol fraction CO_2 in inlet and outlet gases
X	biomass (g/L)
$y_i(k)$	raw dissolved oxygen measurement (% saturation)
$\bar{y}_i(k)$	filtered dissolved oxygen measurement (% saturation)
$Y_{X/gly}$	yield coefficient of biomass on glycerol (g/g)
$Y_{X/asp}$	yield coefficient of biomass on asparagine (g/g)
$Y_{A/gly}$	yield coefficient of antibiotic on glycerol (mg/g)
$Y_{A/asp}$	yield coefficient of antibiotic on asparagine (mg/g)

Subscripts

gly	glycerol
asp	asparagine
abtc	antibiotics
sat	saturation
sp	set point

Greek symbols

μ_l	log–mean circulation time
σ	standard deviation of circulation times, (s)
σ_θ	normalized standard deviation
σ_l	log–mean standard deviation
μ	specific growth rate (h^{-1})

GLOSSARY

aerobe	an organism that grows in the presence of O_2.
antibiotic	a chemical agent produced by one organism that is harmful to other organisms.
catabolism	the biochemical processes involved in the breakdown of organic compounds, usually leading to the production of energy.
chemostat	a bioreactor in which microorganisms are maintained in the experimental phase of growth by continuous addition of fresh medium and removal of effluent.
dilution rate	reciprocal of the residence time of a culture in a bioreactor (flow rate/bioreactor volume).
enzyme	a protein functioning as the catalyst of living organisms, which promotes specific reactions or groups of reactions.
fermenter	a vessel in which fermentations are carried out, also known as bioreactor.
idiophase	secondary metabolite production phase.
lag phase	the period after inoculation of a population before growth begins.
mycelium	the branched network of hyphae which makes up the structure of fungi and some bacteria.
repression	the process by which the synthesis of an enzyme is inhibited by the presence on an external substance, the repressor.

respiration	catabolic reactions producing ATP in which either organic or inorganic compounds are primary electron donors and inorganic compounds are ultimate electron acceptors.
secondary metabolites	metabolites not required for the survival of an organism.
trophophase	growth phase in secondary metabolism without secondary metabolite production.

CHAPTER 1

INTRODUCTION

Streptomyces clavuligerus, a filamentous bacterium, produces four β–lactam compounds : two cephalosporins, penicillin N and clavulanic acid. Currently, β–lactam antibiotics form the largest share of the world's antibiotic market. The β–lactam antibiotics include penicillins such as penicillin G, penicillin V, ampicillin, cloxacillin, and peperacillin; cephalosporins such as cephalothin, cephaloridine, cephalexin, and cefaclor; and cephamycins such as cefoxitin (Demain, 1989). The *Streptomyces* species therefore form an important source for industrial production of β–lactam antibiotics.

Previous studies have investigated the role of carbon source (Aharonowitz and Demain, 1978), nitrogen source (Brana *et al.*, 1986), and phosphates (Lebrihi *et al.*, 1987) in the regulation of antibiotic production in *S. clavuligerus*, using batch shake flasks. At higher growth rates, using easily assimilated carbon sources like glycerol, and nitrogen sources like ammonium salts, separation of the antibiotic production phase (idiophase) from the growth phase (trophophase) is observed. Utilization of complex substrates, such as starch and amino acids, such as asparagine as nitrogen source gives higher yields and growth associated cephamycin C production (Lebrihi *et al.*, 1988). The biosynthetic pathway for antibiotic production relies on the products or intermediates of a primary pathway. The process of antibiotic synthesis by the microorganism is termed secondary metabolism because it is deemed unnecessary for growth of the microorganism. The phenomenon of suppressive action on antibiotic production by carbon/energy sources promoting rapid growth is called 'catabolite repression' (Gallo and Katz, 1972). As an example, Lebrihi *et al.* (1988) showed that cephamycin C production in *Streptomyces clavuligerus* is growth associated during growth on chemically defined basal medium. Excess amounts of glycerol, and to a lesser extent starch, decrease cephamycin C production by the

repression of the biosynthetic enzyme system responsible for production. Although glycerol and starch do not inhibit the enzyme activities, intermediates of the glycolytic pathway (glucose 6–phosphate and fructose 1,6–phosphate) strongly inhibit the biosynthetic enzyme activities. A study on nitrogen regulation of cephalosporin production in *S. clavuligerus* found that asparagine and glutamine were the best nitrogen sources for antibiotic synthesis (Aharonowitz and Demain, 1979). Antibiotic production was poor when cultures were grown on inorganic ammonium salts.

In batch fermentations, the production of antibiotics is generally characterized by a growth phase (trophophase), followed by the production phase (idiophase). Most of the regulation studies have been carried out in batch systems, where the fermentation environment constantly changes with time. Chemostat or continuous culture studies can be used to vary the growth rate and substrate concentrations independently, and evaluate the effect on antibiotic production.

Most industrial antibiotic fermentations are carried out in large stirred fermentors upto 300 m^3 in volume. Due to limitations on power input, aeration and the configuration of the stirred tank, spatial gradients exist in physical properties such as pH, dissolved oxygen (DO), dissolved carbon dioxide, and nutrient levels (Lilly, 1987). The morphology of Streptomyces species is filamentous. Growth results in extensive branching, resulting in highly viscous and non–Newtonian fermentation broths (Allen *et al.*, 1990). High viscosity and non–Newtonian rheology result in result in oxygen transfer problems (Hickman and Nienow, 1986). Detrimental effects on the organisms result if the dissolved oxygen levels fall below a critical value. Poor] erated zones exist in large fermentors, with low dissolved oxygen levels. As a result, the cells are exposed to a continuously fluctuating environment with respect to dissolved oxygen. The effects of such fluctuations on growth and antibiotic production are poorly understood. Oxygen is required in

several reactions in the biosynthetic pathway for cephalosporins in *S. clavuligerus* (Demain *et al.*, 1982). Rollins *et al.* (1988) found an increase in final antibiotic concentrations when dissolved oxygen was controlled at higher values during batch fermentations of *S. clavuligerus* using complex media. In a subsequent study using a defined medium, lower antibiotic yields were obtained when dissolved oxygen was maintained at saturation levels during the batch fermentation (Rollins *et al.*, 1989). Hence, dissolved oxygen is also an important parameter involved in regulation of antibiotic synthesis. Knowledge about the effect of dissolved oxygen fluctuations, and controlled dissolved oxygen levels on growth and antibiotic production can be used for fermentation scale–up and optimization.

The importance of CO_2 in microbial fermentations and the detrimental effects of elevated pCO_2 levels on growth and metabolism have been recognised. (Ho *et al.*, 1987 ; Jones *et al.*, 1982). There is a lack of on–line measurement techniques for biomass, and product in fermentations. Parameters such as oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and respiratory quotient (RQ, the ratio of CER to OUR) have been used as indicators of the physiological state of the culture. The dynamic equilibrium of CO_2 in aqueous systems is affected by pH and pressure. CER, and hence RQ, is sensitive to pH changes in the broth and changes in fermentor head space pressure. These effects of pH and pressure on CER and RQ have not been considered in any of the studies in the literature.

The main objectives of this thesis are :

(1) To develop an experimental strategy for studying the effects of large scale operation on microbes using small scale equipment.

(2) To use the developed strategy to study the effects of dissolved oxygen fluctuations experienced in large fermentors on growth and antibiotic production in *Streptomyces clavuligerus*.

(3) To study the effect of constant and stepwise controlled dissolved oxygen levels

on growth and antibiotic production in *S. clavuligerus*.

(4) To develop a mathematical model for hydration of CO_2 in fermentors.

Chapter 2 of this thesis is a preliminary study that deals with the use of specific growth rate as a parameter for comparison of batch and chemostat data for antibiotic production. Stable antibiotic production in chemostat cultures was obtained and growth and antibiotic production data from batch and chemostat experiments were compared in the same range of specific growth rates. The effects of a change in the partial pressure of oxygen in the aeration stream on growth and antibiotic production in defined media batch fermentations of *S. clavuligerus* is studied in chapter 3. Chapter 4 investigates the effect of dissolved oxygen controlled at constant levels, and step control during trophophase and idiophase, on growth and antibiotic production in *S. clavuligerus*. A Monte Carlo method, to simulate the DO fluctuations in a large fermentor using a laboratory fermentor is presented in chapter 5, along with an application of this method to *Saccharomyces cerevisiae* (baker's yeast). The application of the Monte Carlo method to study the effect of DO fluctuations on *S. clavuligerus* is discussed in chapter 6. Chapter 7 presents experimental data and a verified model to describe the pH and pressure effects on the kinetics of carbon dioxide hydration in fermentors. The calibration curves for the various analytical methods used are given in Appendix A. The data from repeated experiments are given in Appendices B and C.

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CHAPTER 2

CELL GROWTH AND ANTIBIOTIC PRODUCTION IN BATCH AND CHEMOSTAT CULTURES OF *STREPTOMYCES CLAVULIGERUS*

Introduction

Antibiotic biosynthesis is regulated by carbon source, nitrogen source, phosphate source and trace elements. The production of antibiotics is characterized by growth dissociated synthesis, where the production phase (idiophase) follows a phase of rapid growth (trophophase).

In batch cultures, the fermentation environment constantly changes with time, making it difficult to establish the mechanism responsible for trophophase–idiophase kinetics. In steady–state chemostat cultures, since the substrate and nutrient concentrations can be varied independent of the growth rate, the regulatory effects of these parameters can be studied. Production of antibiotics in chemostat cultures can show diverse behaviours. Antibiotic production can be negatively correlated with specific growth rate e.g. thienamycin and cephamycin C production in *Streptomyces cattleya* (Lilley *et al.*, 1981), and *Streptomyces clavuligerus* (Lebrihi *et al.*, 1988). In other studies production was growth associated, eg. oxytetracycline production by *Streptomyces rimosus* (Rhodes, 1984), production of erythromycin A and its precursors by *Streptomyces erythraeus* (Trilli *et al.*, 1987), and chlortetracycline production by *Streptomyces aureofaciens* (Siktya *et al.*, 1961). The production of penicillin G by *Penicillium chrysogenum* was proportional to specific growth rate up to a limiting value (0.01–0.014 h^{-1}) (Ryu *et al.*, 1980). Tylosin production by *Streptomyces fradiae* was maximal at a dilution rate of 0.017 (h^{-1}) (Gray *et al.*,1980). These different behaviours reflect the different mechanisms by which antibiotic synthesis is regulated by growth rate and medium constituents. These studies did not compare data from batch and chemostat experiments.

The purpose of this study was to compare antibiotic production in batch and chemostat cultures over a common range of specific growth rates. *Streptomyces clavuligerus* was selected as a test organism. It produces four β – lactam compounds, two cephalosporins, penicillin N, and clavulanic acid. The carbon regulation (Aharonowitz *et al.*, 1978), nitrogen regulation (Brana *et al.*, 1985), and phosphate regulation (Lebrihi *et al.*,1987) of cephalosporin production in this organism have been reported. In this study, batch and chemostat experiments were carried out using a chemically defined medium. The effects of specific growth rate on biomass, protein content, and total antibiotics (penicillin N and cephamycin C) were studied.

Materials and Methods

Organism and media

The organism used in this study was *Streptomyces clavuligerus* NRRL 3585. The same chemically defined medium was used for both batch and chemostat cultures : 0.5 % glycerol, 0.4 % L–asparagine, 0.025 % $MgSO_4$.$7H_2O$, 0.069 % K_2HPO_4, 0.031 % KH_2PO_4, and 1 mL of trace salts solution (containing 100 mg of $FeSO_4$.$7H_2O$, 100 mg of $MnCl_2$.$4H_2O$, 100 mg of $ZnSO_4$.$7H_2O$ and 130 mg of $CaCl_2$.$2H_2O$ per 100 mL of water) per 1 litre of medium. Previous work on carbon and nitrogen source regulation (Aharonowitz and Demain, 1978, 1979) showed better antibiotic biosynthesis for the glycerol, asparagine medium compared to other defined media. This medium was carbon limiting.

Batch cultures

Spore stocks of *S. clavuligerus* were maintained in glycerol at –60 ^{0} C. A 2 mL volume of the spore stock was inoculated into 100 mL of seed medium containing 1% glycerol, 2 % sucrose, 0.5 % tryptone, 0.1 % yeast extract, 1.5 %phytone and 0.02 % K_2HPO_4.The inoculated medium was agitated at 260 rpm in a 250 mL Erlenmeyer flask for 24 hrs at 28^0 C. Samples of 6 mL each from the primary seed culture were used to inoculate 250 mL Erlenmeyer flasks containing 100 mL of seed medium. These flasks containing the secondary seed culture were agitated at 28^0 C for 24 hrs at 260 rpm to prepare the inoculum for the batch fermentor. One litre of the secondary seed culture was used to inoculate 50 l of fermentation medium (2 % v/v) in a LH 2000 series fermentor. The fermentor was agitated at 300 rpm and aerated with 30 l/min of air.

Chemostat cultures

The chemostat experiments were carried out in a NBS Bioflo Model C30 with a working volume of 400 mL. A 0.5 mL of the spore stock was inoculated into 25 mL of the same seed medium as used for the batch culture. The inoculated medium was agitated at 28^{o} C for 24 hrs at 260 rpm to prepare the inoculum for the chemostat. A 2 % v/v of this seed culture was used to inoculate 400 mL of fermentation medium in the chemostat. Medium flow was started 24 hrs after inoculation and was supplied by means of an LKB peristaltic pump and a constant volume was maintained with an overflow weir. The chemostat was agitated at 600 rpm and aerated with 500 mL/min of air.

The temperature and pH were controlled in the batch and chemostat cultures at 27^{o} C and 6.8 respectively. Samples were taken at intervals for biomass, protein, glycerol, asparagine, and antibiotic analysis.

Analytical methods

Biomass concentrations were measured by filtering a known volume of sample and drying it to a constant weight at 100^{o} C. The optical density (OD) of the samples were also measured at 660 nm in a LKB Novaspec 4049 spectrophotometer. The OD was linearly related to dry cell weight (DCW) throughout the growth phase (Table A.1).

Total cell proteins were measured using the BioRad method (Bradford, 1976).

Total cephalosporins produced were measured by the agar plate diffusion assay (Brana *et al.*, 1985) using *E.coli* ESS as the indicator organism, with cephalosporin C as standard. The standard curve was found to be exponential (Figure A.1).

Glycerol was determined by gas chromatography using a Hewlett Packard 5890A Gas Chromatograph. The column used was a crosslinked Megabore, 10m. x0.53mm x 2.0μm film thickness with 50 % phenyl methyl silicone. The internal standard used was n–hexanol with tetrahydrofuran as the solvent (Table A.2).

Asparagine was determined using a ninhydrin assay (Nivard and Tesser, 1965). (Table A.2).

Estimation of specific rates and yield coefficients

The batch and chemostat data were compared for specific growth rates of 0.013 to 0.029 h^{-1}.The data for chemostat cultures were used to obtain steady–state yield coefficients and estimates of standard error. The data from batch experiments were interpolated at 2–h intervals using a third–order spline, differentiated, and divided by the biomass concentration to obtain the specific production rates of biomass and antibiotics, and the specific utilization rates of glycerol and asparagine. The differential yields from the batch culture were defined as follows :

$$Y_{A/X} = \frac{dA}{dX} \qquad (2.1)$$

where A = antibiotic concentration, X = biomass concentration, or glycerol consumed, or asparagine consumed.

A plot of antibiotic concentration versus X was linear in the range of interest, with slope of $Y_{A/X}$. Hence the yield coefficients were obtained by linear regression, and standard errors were estimated for batch culture using the t–distribution.

Results and Discussion

Figure 2.1 illustrates the time profiles of biomass (DCW), total antibiotics, glycerol and asparagine for the batch run. The biomass profile shows the phases of lag, exponential growth and lysis. Antibiotic production starts at about 15 h, during the growth phase, though the maximum antibiotic titre of 22 mg/l is obtained in the phase of decline. As shown by the arrows, the range of comparison with chemostat data (based on equivalent μ values) accounts for only a narrow region, preceding the stationary phase, and does not include the high antibiotic production phase. A plot of specific rate of antibiotic production as a function of specific growth rate shows a maximum (Figure 2.2). This maximum corresponds to the early growth phase when low DCW and antibiotic levels are observed. The biosynthetic machinery responsible for antibiotic production is set up early in the fermentation and is effective well into the decline phase. There is evidence for such early establishment, in the case of nitrogen regulation of the biosynthetic pathway (Aharonowitz and Demain, 1979).

For this run it was not possible to obtain a single constant value for the yield coefficient of biomass on glycerol, $Y_{X/gly}$, however, two different values of 0.22 and 1.23 (g DCW/g glycerol) can be used to describe the growth. The two values roughly correspond to the phases of growth and antibiotic production respectively. A constant value of 1.8 (g DCW/g asparagine) was obtained for $Y_{X/asp}$, the yield coefficient of biomass on asparagine.

Figure 2.3 shows data from a typical chemostat run. The various parameters are plotted as a function of dimensionless retention time (dilution rate (h^{-1}) x time (h)). As shown here, in the chemostat runs, little glycerol was detected in the outlet. Fluctuations were observed in the antibiotic concentration even when other quantities had reached constant values, which can be attributed largely to the precision of the bioassay. The data in Table 2.1 illustrate the variation of DCW and

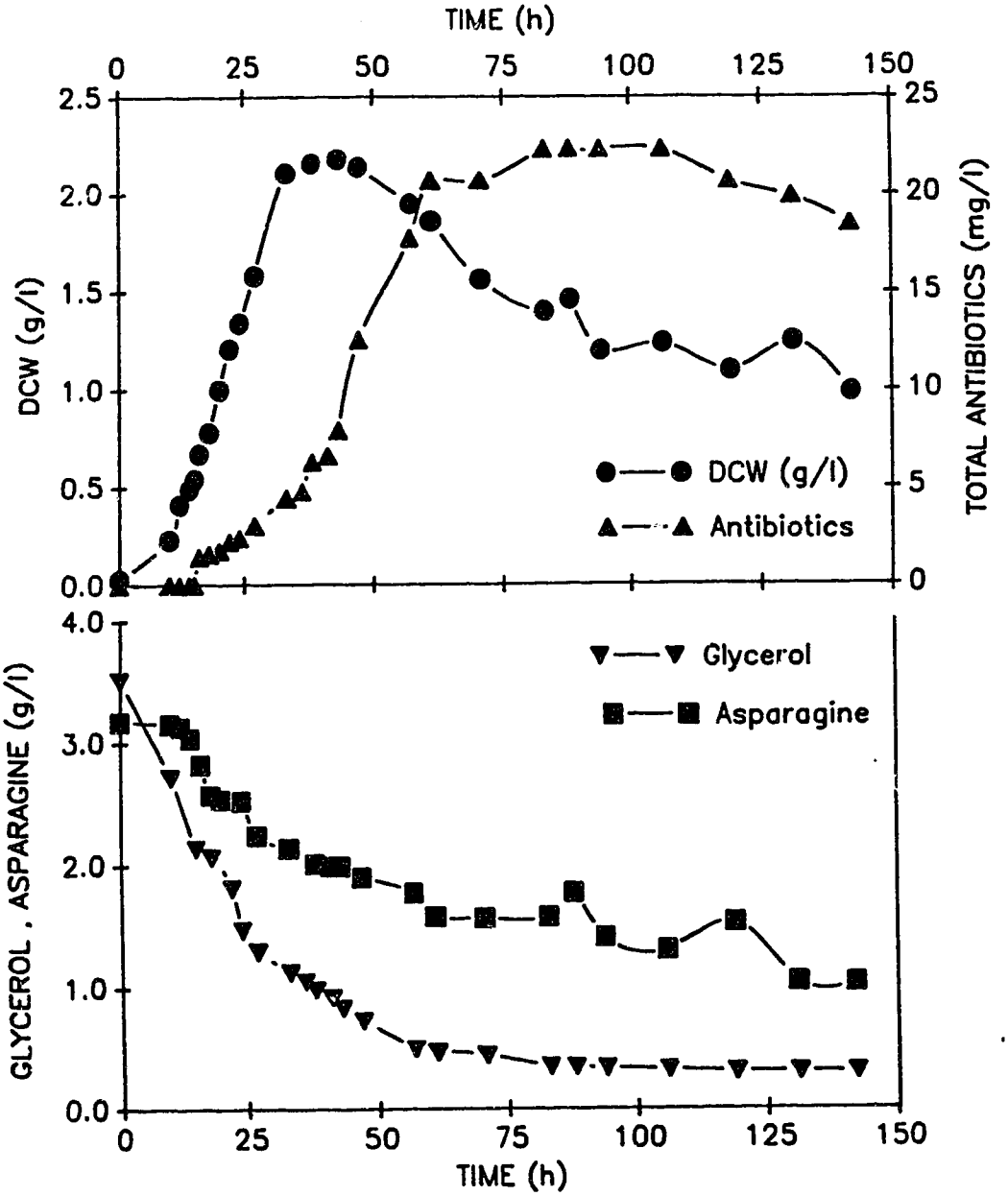

Figure 2.1 Time profiles of DCW, total antibiotics, glycerol and asparagine for batch fermentation.

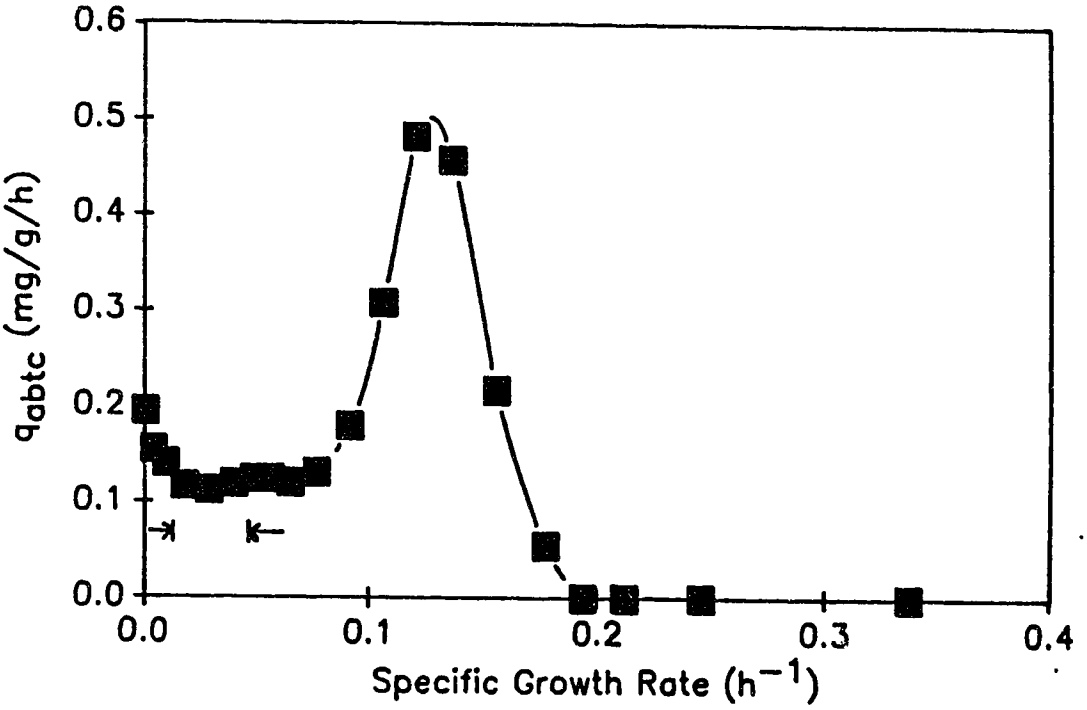

Figure 2.2 Specific rate of antibiotic production plotted as a function of specific growth rate for the batch run.

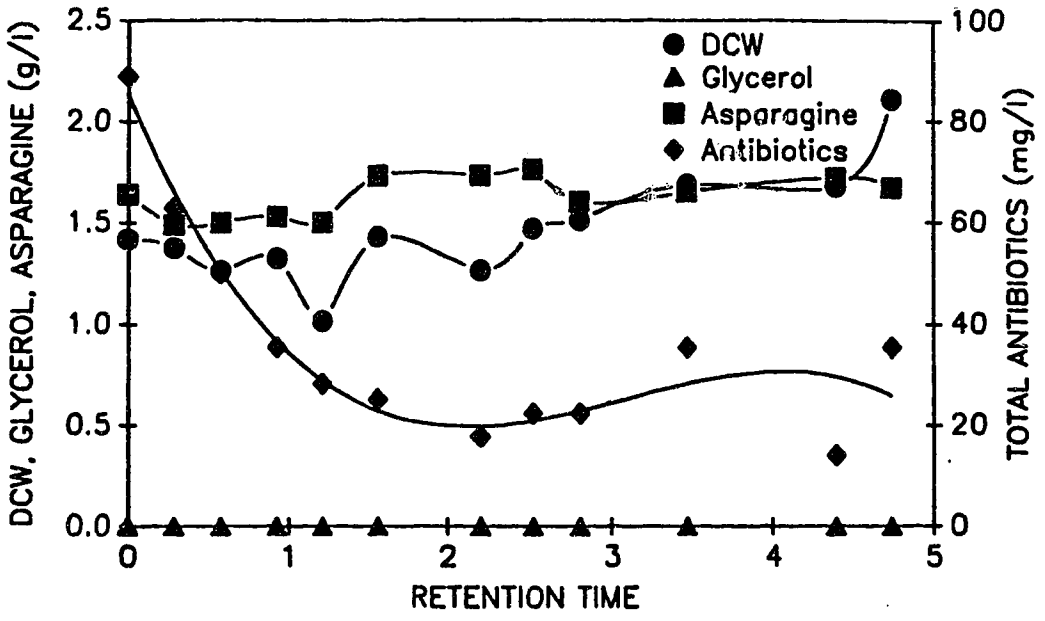

Figure 2.3 Time profiles of total antibiotics, DCW, glycerol and asparagine for a chemostat run.

antibiotics with dilution rate. All error limits correspond to 95 % confidence interval estimates. With increasing dilution rates, a decrease in DCW is accompanied by a decrease in total antibiotic concentration. However, the specific production rate is constant or shows a small increase in the range 0.2 to 0.6 (Figure 2.4). In a recent study on chemostat cultures of S. *clavuligerus* by Lebrihi *et al.* (1987), specific cephamycin C production (mg Ceph C /g DCW /h) decreased from 0.6 to 0.2 when the dilution rate was increased from 0.01 to 0.05 h^{-1}. As shown in Table 2.2, the yield coefficients for biomass and antibiotics decrease with increasing D, except for $Y_{X/gly}$ which goes through a maximum. Specific uptake rates q_{gly} and q_{asp} increase with D (Figure 2.4). In the production of oxytetracycline in chemostat cultures of S. *rimosus* $q_{glucose}$ and $q_{nitrogen}$ increased with increasing D, and the specific production rate also increased with increasing D for carbon and phosphorus limited cultures (Rhodes, 1984). Such an increase in specific uptake rates of C and N source with D has also been reported in chemostat cultures of *S. cattleya* (Lilley *et al.*, 1981), but was accompanied by a decrease in q_{abtc}. The increased specific uptake rates may be responsible for the reduction in antibiotic concentrations. Similar reduction in antibiotic production has been reported as a result of increased ammonium concentrations (Brana *et al.*, 1985) and increased glycerol concentrations (Aharonowitz, and Demain, 1978) in the medium during batch cultures of *S. clavuligerus*.

Antibiotic yield coefficients and specific rate values from batch cultures, corresponding to μ values ranging from 0.013 to 0.029 h^{-1} (the range of D values for chemostat experiments) are shown in Tables 2.1, and 2.2 and Figure 2.4 for comparison with values from chemostat runs. In the range of comparison, the batch data show higher DCW and lower antibiotic concentrations (Table 2.1). The specific production rate, q_{abtc} and the specific utilization rates q_{gly} and q_{asp} are also lower

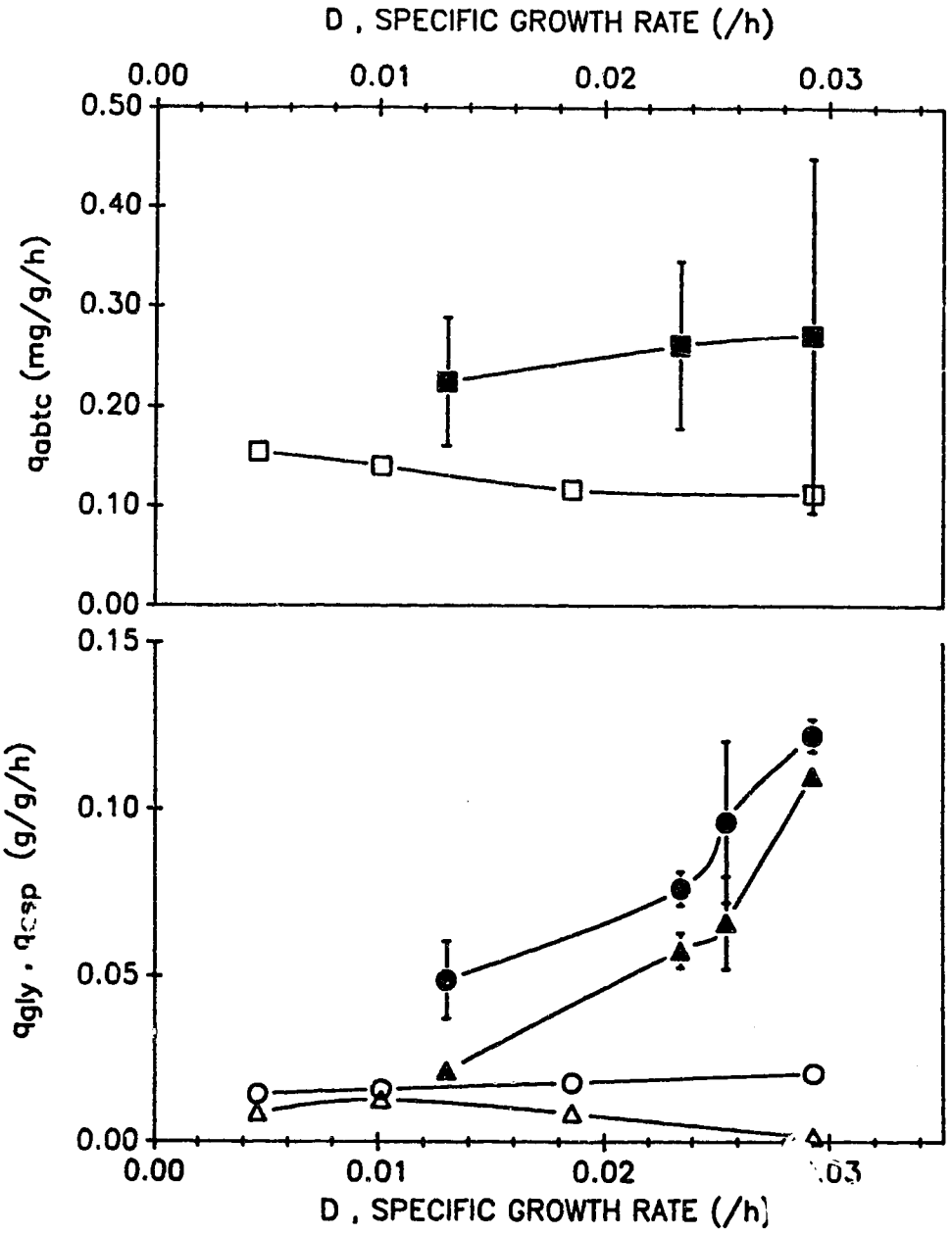

Figure 2.4 Comparison of specific antibiotic production rat... and q_{abtc} chemostat (■), specific glycerol utiliz... batch (O) and q_{gly} chemostat (●), specific aspara... rates, q_{asp} batch (Δ) and q_{asp} chemostat (▲).

Table 2.1 Dry cell weight and total antibiotics from batch and chemostat experiments in the same range of specific growth rates.

D (h^{-1})	DCW (g/L)	Antibiotics (mg/L)
0.013	1.52 ±0.14	25.77 ±7.97
0.023	1.32 ±0.11	14.33 ±4.25
0.029	1.02 ±0.05	9.44 ±7.24
Batch data	2.1 ±0.11	4.89 ±0.99

for the batch run, but show values which approach the rates in the chemostat at low growth rates (Figure 2.4).

Table 2.2 shows yield coefficients from batch and chemostat runs. Over the entire range of dilution rates, $Y_{X/gly}$ values from the chemostat (0.24 – 0.31 g/g) are lower than the yield coefficient from the batch run (1.11 g/g). The value for $Y_{X/asp}$ from batch culture agrees well with the corresponding coefficient from continuous cultures. Within error bounds, the antibiotic yield coefficient, and $Y_{A/asp}$ from batch and chemostat runs are of similar magnitude. The same degree of agreement is not observed for $Y_{A/gly}$. The agreement for both yield coefficients is better near the lower limit of the dilution rates, where the DCW from chemostat data is numerically comparable to that from batch data. In the range of comparison, the protein fraction in DCW was 0.12 for the batch run and 0.43 – 0.79 for the chemostat runs (increasing with increasing D). During the entire growth phase the average protein fraction in DCW for the batch run was 0.30.

In growth dissociated antibiotic production in batch and chemostat cultures, no definite overlap was observed between yield coefficients for comparable specific growth rates. As the dilution rate approaches zero in the chemostat, the yield coefficients and the specific production and uptake rates approached the batch case for comparable specific growth rates. Protein content of the cells was different in the batch and chemostat cases. The higher protein content in chemostat culture could be in response to higher specific uptake rates, q_{asp} and q_{gly}.

Table 2.2 Comparison of yield coefficients from batch and chemostat experiments in the same range of specific growth rates.

D (h^{-1})	$Y_{X/gly}$ (g/g)	$Y_{X/asp}$ (g/g)	$Y_{abtc/gly}$ (mg/g)	$Y_{abtc/asp}$ (mg/g)
.013	0.29 ±0.08	.61 ±0.06	5.15 ±1.6	10.28 ±2.98
.023	0.31 ±0.02	0.41 ±0.05	3.36 ±1.00	4.43 ±1.26
.029	.24 ±0.01	.27 ±0.01	2.22 ±1.82	2.46 ±1.9
Batch data	1.11	0.63	12.21 ±5.18	7.61 ±3.15

Conclusions

In batch cultures of *S.clavuligerus*, antibiotic production begins in the growth phase, though most of the production is growth dissociated. Continuous, stable antibiotic production is obtained in chemostats with a small increase in specific antibiotic production with dilution rate. The yield coefficients from batch and chemostat data approach similar values at lower comparable specific growth rates (D, $\mu \rightarrow 0$). Nutrient limitations in chemostat can change the cellular compositon dramatically. In such cases μ alone is not an appropriate indicator of metabolism, and cannot be used by itself for comparing performance of batch and chemostat modes of operation for secondary metabolite production.

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CHAPTER 3

EFFECTS OF REDUCED OXYGEN ON GROWTH AND ANTIBIOTIC PRODUCTION IN *STREPTOMYCES CLAVULIGERUS*[1]

Introduction

The biosynthesis of antibiotics is normally characterized by a phase of rapid growth (trophophase) followed by the phase of antibiotic production (idiophase). Their production is strongly influenced by the nature and concentration of the carbon source, nitrogen source, phosphorus source, and trace elements. The regulation of secondary metabolism has been reviewed recently (Drew and Demain, 1977 ; Aharonowitz and Cohen, 1986).

This study reports the results of investigation on the effect of oxygen levels on growth and antibiotic production by *S. clavuligerus* in a defined medium. This organism is a filamentous bacteria (Higgens and Kastner, 1971) which produces four β–lactam compounds, two cephalosporins, penicillin N and clavulanic acid. The carbon regulation (Aharonowitz and Demain, 1978), nitrogen regulation (Brana *et al.*,1986), and phosphate regulation (Lebrihi *et al.*,1987) of cephalosporin production in this organism have been reported. The possible involvement of aeration (Brana *et al.*, 1983) and protein synthesis (Hu *et al.*, 1984) in carbon source regulation of antibiotic synthesis has been investigated. Most of the regulation studies have been restricted to shake flask experiments.

In stirred fermentors, the oxygen transfer to microbial cells is affected by the aeration rate, the agitation rate and broth characteristics. The morphology of

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[1]A version of this study was published earlier in *Biotechnology Letters* (Yegneswaran, *et al.*, 1988).

filamentous organisms such as *Streptomyces* is affected by shear forces resulting from agitation. The effect of such shear on antibiotic production is not known (Drew and Demain, 1977). The pCO_2 level in the fermentation medium also is a function of the oxygen transfer and the respiration rate of cells. Thus the effects of pO_2, shear, and pCO_2 may interact to affect growth and antibiotic production.

In this study the proportion of oxygen in the inlet air stream is varied while the total gas flow into the batch fermentor and the agitation rate are maintained at constant values. This protocol changed the pO_2, and by inference pCO_2, in the fermentor without altering the effect of shear forces on mycelium formation. The effect of reduced oxygen levels on antibiotic synthesis, protein synthesis, growth, substrate and nutrient utilization is studied in a batch fermentor using chemically defined media.

Materials and Methods

S. clavuligerus NRRL 3585 was used in this study. The chemically defined medium contained 0.5 % glycerol, 0.4 % L–asparagine, 0.025 % $MgSO_4$.$7H_2O$, 0.069 % K_2HPO_4, 0.031 % KH_2PO_4, 1 mL of trace salts solution previously described (Aharonowitz and Demain, 1978) per 1 litre of medium. A 2 mL volume of seed culture obtained from glycerol spore stocks was inoculated into 100 mL of seed medium (1% glycerol, 2 % sucrose, 0.5 % tryptone (Difco), 0.1 % yeast extract (Difco), 1.5 %phytone (Becton and Dickinson) and 0.02 % K_2HPO_4) in a 250 mL Erlenmeyer flask. The inoculated medium was incubated 28^0 C for 24 hours on a rotary shaker. Six–mL samples from seed cultures were used to inoculate 250 mL Erlenmeyer flasks containing 100 mL of seed medium. These flasks also were agitated at 28^0 C for 24 hours on a rotary shaker to prepare the inoculum for the fermentor. One litre of the seed culture was used to inoculate 50 l of fermentation medium (2 % v/v) in an LH 2000 series fermentor. The initial pH of all media was adjusted to 6.8 with NaOH or HCl. The fermentor was agitated at 300 rpm and the temperature and pH were controlled at 27^0 C and 6.8 respectively. The pH, pO_2, temperature and redox potential (eH) were recorded every hour. Samples were taken at intervals for biomass, protein, glycerol, asparagine, and antibiotic analysis. The fermentor was aerated with 30 l/min of air. For the run with reduced oxygen, a mixture of 20 l/min of air and 10 l/min of nitrogen was used, keeping the total gas flow constant. Thus, the fermentor was aerated with 21 % oxygen and 14 % oxygen during the control fermentation and the reduced oxygen fermentation respectively.

Biomass concentrations were measured by filtering a known volume of sample and drying it to a constant weight at 100^0 C. The absorbance of samples was measured at 660 nm in a LKB Novaspec 4049 spectrophotometer. The absorbance was linearly related to dry cell weight upto 2.75 g/l DCW.

Total cephalosporins produced were measured by the agar plate diffusion

assay (Brana *et al.*,1985) using *E.coli* ESS as the indicator organism, with cephalosporin C as standard.

For protein determination, cells were hydrolysed with 4 N NaOH at 45^{0}C for 2.5 hours, then neutralized with HCl. Protein was determined using the Bio–Rad Protein Assay (Bradford, 1976).

Glycerol was determined by gas chromatography using a Hewlett Packard 5890A Gas Chromatograph. The column used was a crosslinked Megabore, 10m x 0.53mm x 2.0μm film thickness with 50 % phenyl methyl silicone. The internal standard used was n–hexanol with tetrahydrofuran as the solvent.

Asparagine was determined using a ninhydrin assay (Nivard and T..sser, 1965). The ninhydrin reagent was prepared by adding 2 g of 1,2,3,–triketohydrindene, 0.2 g hydrindantin, to 75 mL of methyl cellusolve, and 25 mL 4 N Na acetate buffer (pH 5.5). One drop ot 1 N NaOH was added to 1 mL of sample, and evaporated to dryness in an oven overnight. One millilitre each of water and ninhydrin reagent were added to the sample and boiled for 15 minutes. 50 % ethanol was added to the cooled sample and absorbance was read at 570 nm.

Results and Discussion

The data in Figure 3.1 show the DCW and antibiotic profiles for the control fermentation and for the fermentation with 14 % oxygen. The biomass curves comprise a lag, a growth, a short stasis and a prominent lytic phase. Low levels of antibiotic production begin during the growth phase, at about 15h, and the bulk of production occurs later. At reduced oxygen levels no production occurs after the stationary phase. The maximum DCW and antibiotic concentrations in the control run were 2.18 g/l and 22.34 mg/l respectively. The corresponding values for the reduced oxygen level were 2.76 g/l and 17.74 mg/l respectively. The peaks in the DCW occurred at about 32 h in both cases and maximum specific growth rates were 0.34 and 0.36 (/h) for the control run and the run with lower oxygen respectively.

Several reactions in the biosynthetic pathway for cephalosporins have a requirement for oxygen (Demain *et al.*, 1982). Since higher DCW was observed with lower oxygen, the competition for oxygen between cell growth, maintenance, and antibiotic biosynthesis could result in reduced antibiotic concentrations (Brana *et al.*, 1983).The increase in DCW when oxygen was reduced was at most 25 % and was sustained over a series of samples. Even with oxygen reduced to 14 % of the inlet gas, the oxygen saturation never fell below 40 % . This observation suggests that oxygen supply was not growth limiting; rather that high oxygen levels inhibited growth. Hence the optimal oxygen level for biomass formation may be distinctly different from the requirements for antibiotic formation.

Degradation of antibiotics is prominent in the reduced oxygen fermentation after about 50 h, when pO_2 values have stabilized (Figure 3.4) and most of the substrate is exhausted (Figure 3.2). Such degradation was not observed in other *S. clavuligerus* fermentations at lower total air flow rates of about 12 l/min. Since

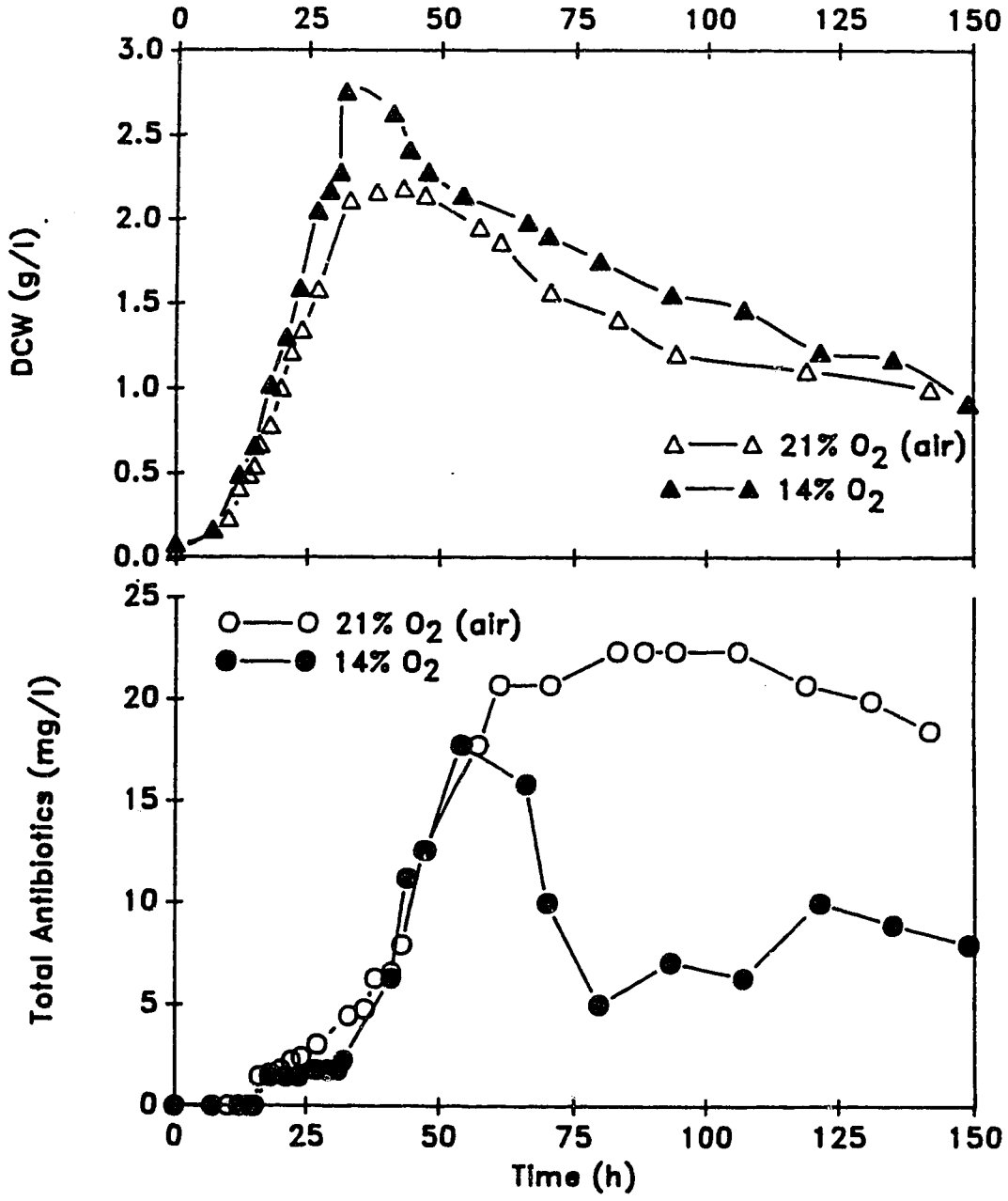

Figure 3.1 Time profiles of biomass (dry cell weight) and total antibiotics.

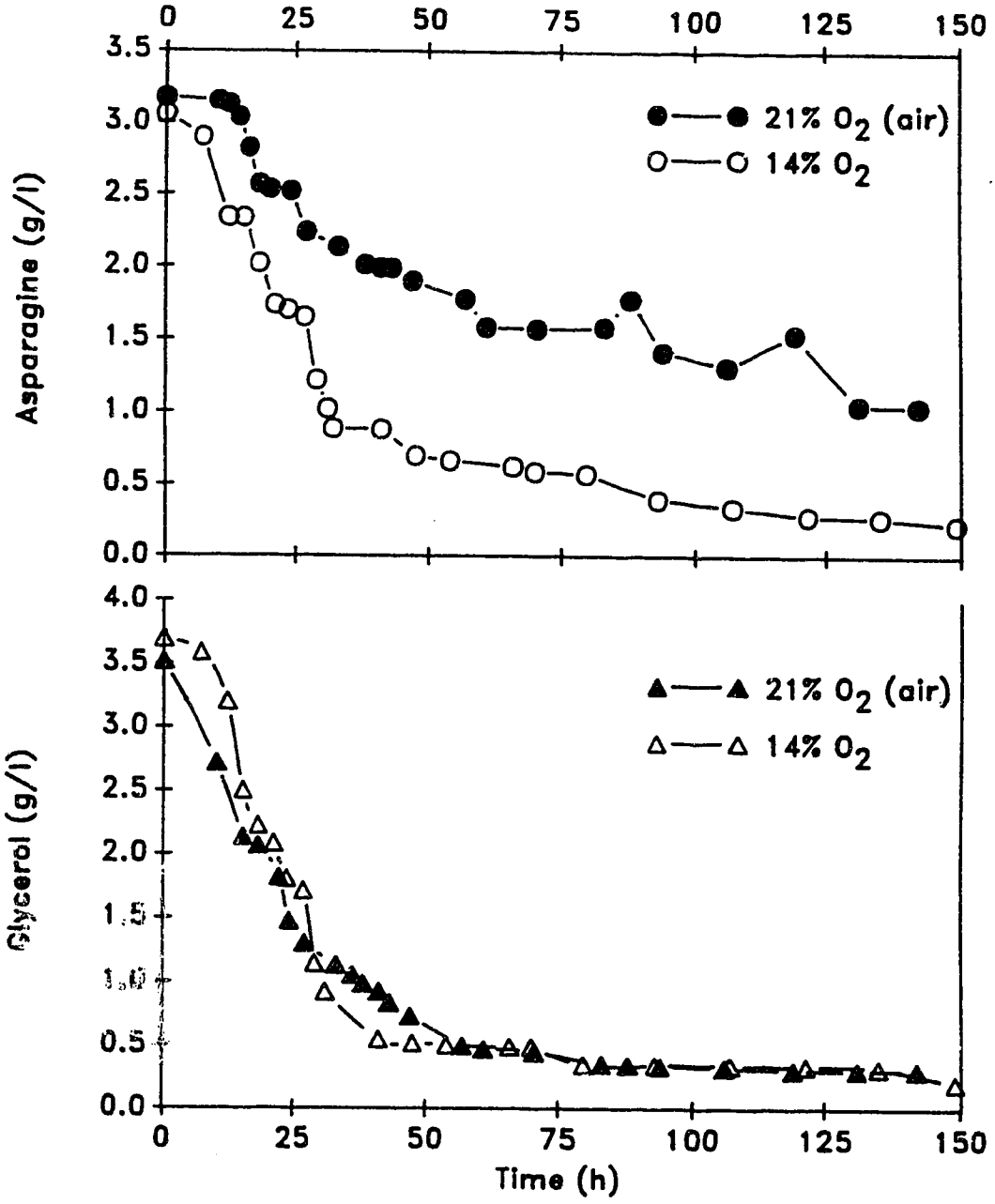

Figure 3.2 Time profiles of glycerol and asparagine.

reduction of air supply alone does not give such a loss of antibiotic, enhanced stripping of carbon dioxide relative to oxygen dissolution may be responsible. A combination of substrate exhaustion, reduced pO_2, and possibly reduced pCO_2 is likely responsible for derepression of hydrolytic enzymes. High levels of carbon dioxide are known to inhibit metabolic processes (Ho *et al.*, 1987), therefore a reduction in pCO_2 by stripping could derepress synthesis of hydrolytic enzymes. Hydrolysis is the most likely cause of the rapid decline in antibiotic concentration because a loss of biosynthetic activity would only eliminate further accumulation.

The data in Figure 3.2 show the utilization of glycerol and asparagine with time. The glycerol concentration follows similar trends in both runs. The total asparagine utilization and the rate of utilization are higher for the run with reduced oxygen. Higher DCW in this run could be a result of carbon being obtained by catabolism of asparagine. Higher asparagine utilization provides for higher basicity of broth, which is reflected in higher acid consumption to maintain pH in the reduced oxygen case, as shown in Figure 3.3. Higher protein levels are observed throughout the course of the reduced oxygen run (Figure 3.3) which can be attributed to the corresponding high asparagine consumption. At the end of the growth phase, proteins accounted for 29% and 39% of the DCW for the control fermentation and the fermentation with lower oxygen levels respectively. Protein synthesis may be responsible for carbon source regulation of antibiotic production (Hu *et al.*, 1984). Lower specific antibiotic yields based on proteins and biomass are obtained in the reduced oxygen case even before hydrolysis, prior to 50 hours. For example, at 48 hours the specific yields (mg antibiotic / g DCW) were 6.2 and 5.5 for the control run and the run with lower oxygen levels respectively. Although higher protein levels are observed with reduced oxygen, the lower yield of antibiotic yield per unit biomass and protein prior to hydrolysis suggests that key biosynthetic enzymes are either repressed or inhibited due to lack of oxygen or that the antibiotic

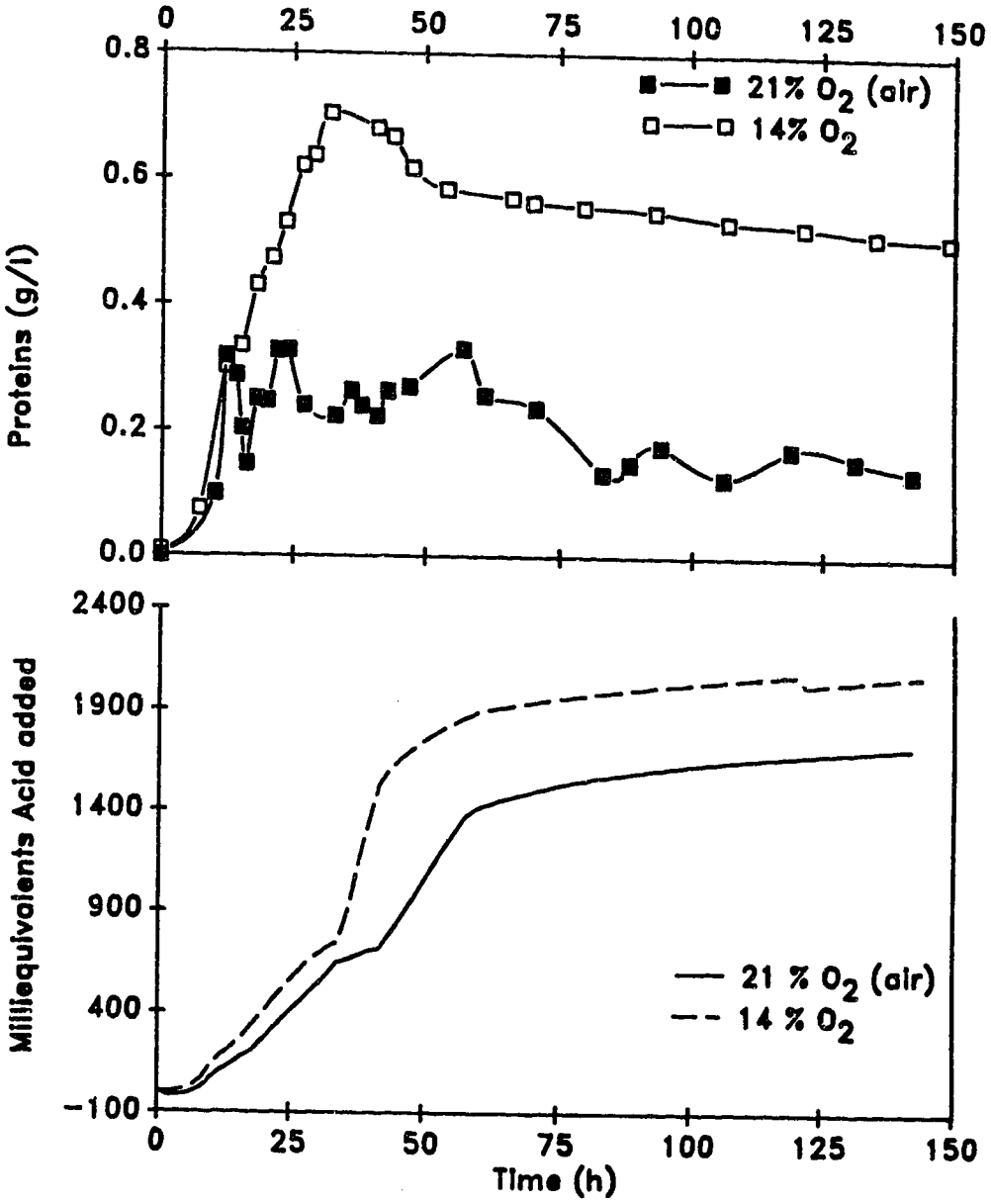

Figure 3.3 Time profiles of proteins and acid addition.

precursors are unavailable. Some recent studies on *S. clavuligerus* grown on maltose (Rollins *et al.*, 1988) and modified trypticase soy broth supplemented with starch (Rollins *et al.*, 1990) showed a reduction in the specific activity of the ring–expansion enzyme, deacetoxycephalosporin C synthase and antibiotic production at reduced dissolved oxygen levels.

The redox potential (eH) follows similar trends with time (Figure 3.4) as pO_2. In the control run, the increase in pO_2 value above 100% at about 100 h, well after the growth phase, was due to a stability problem with the sensor. The drop in eH to a relatively constant value occurs at about 50 h, at about the time when substrate is exhausted, in agreement with an earlier description of such phenomena (Kjaergaard and Joergensen, 1980).

The oxygen utilization rate, OUR and the oxygen utilization rate per unit DCW, OUR/X are shown upto 60 h in Figure 3.5. After 60 h these values remained constant. OUR was estimated as

$$OUR = C\,(pO_{2\,sat} - pO_2)/pO_{2\,sat} \qquad (3.1)$$

where C is a constant.

The larger OUR values for the 14% oxygen run are consistent with the observed enhancement in DCW, relative to the control run. Growth is clearly not limited by oxygen in this concentration range. Both runs give maximal OUR/X values in the early growth phase, at about 10 h. The OUR values level off to constant values after about 50 h.

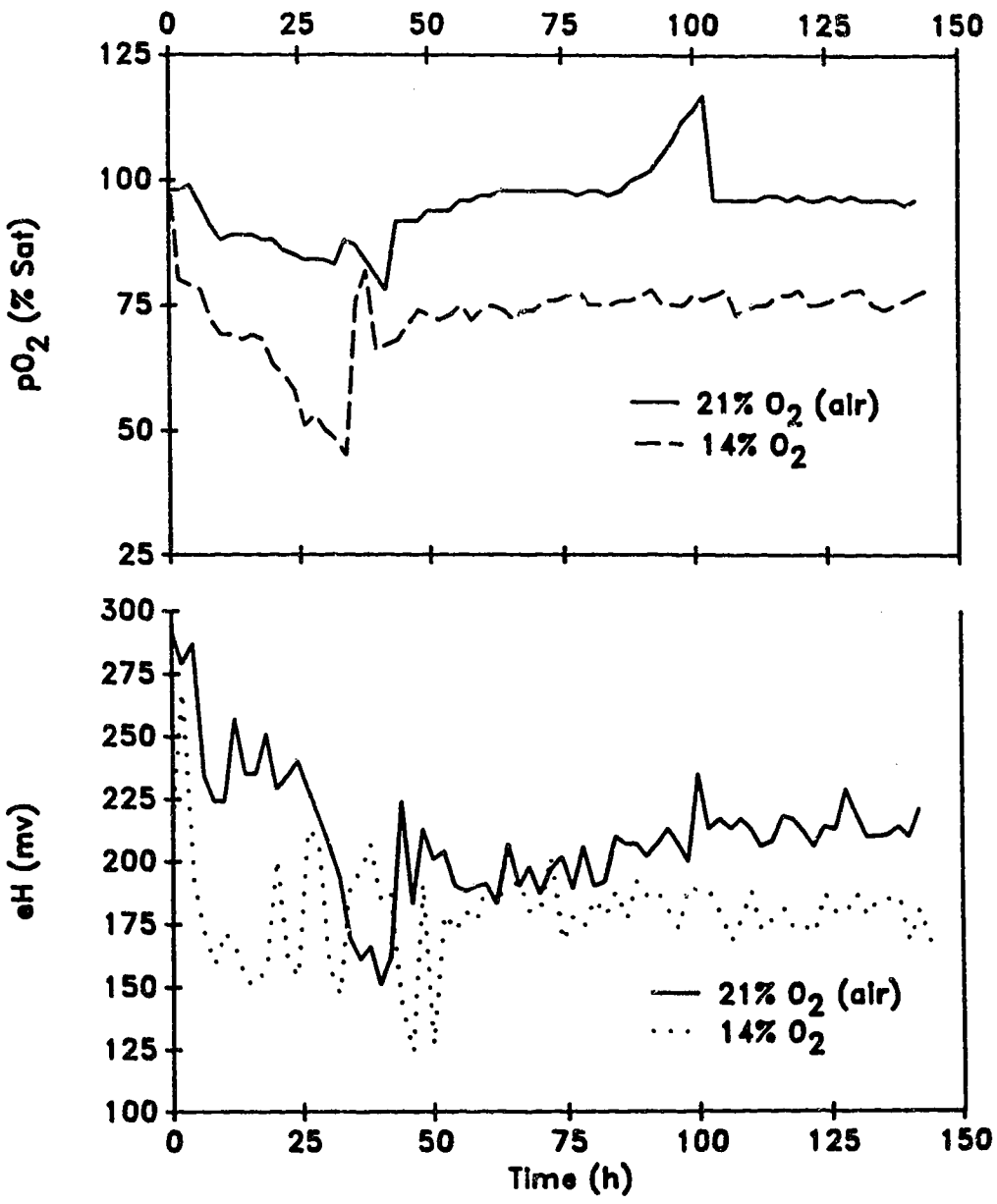

Figure 3.4 Time profiles of pO_2 and redox potential (eH).

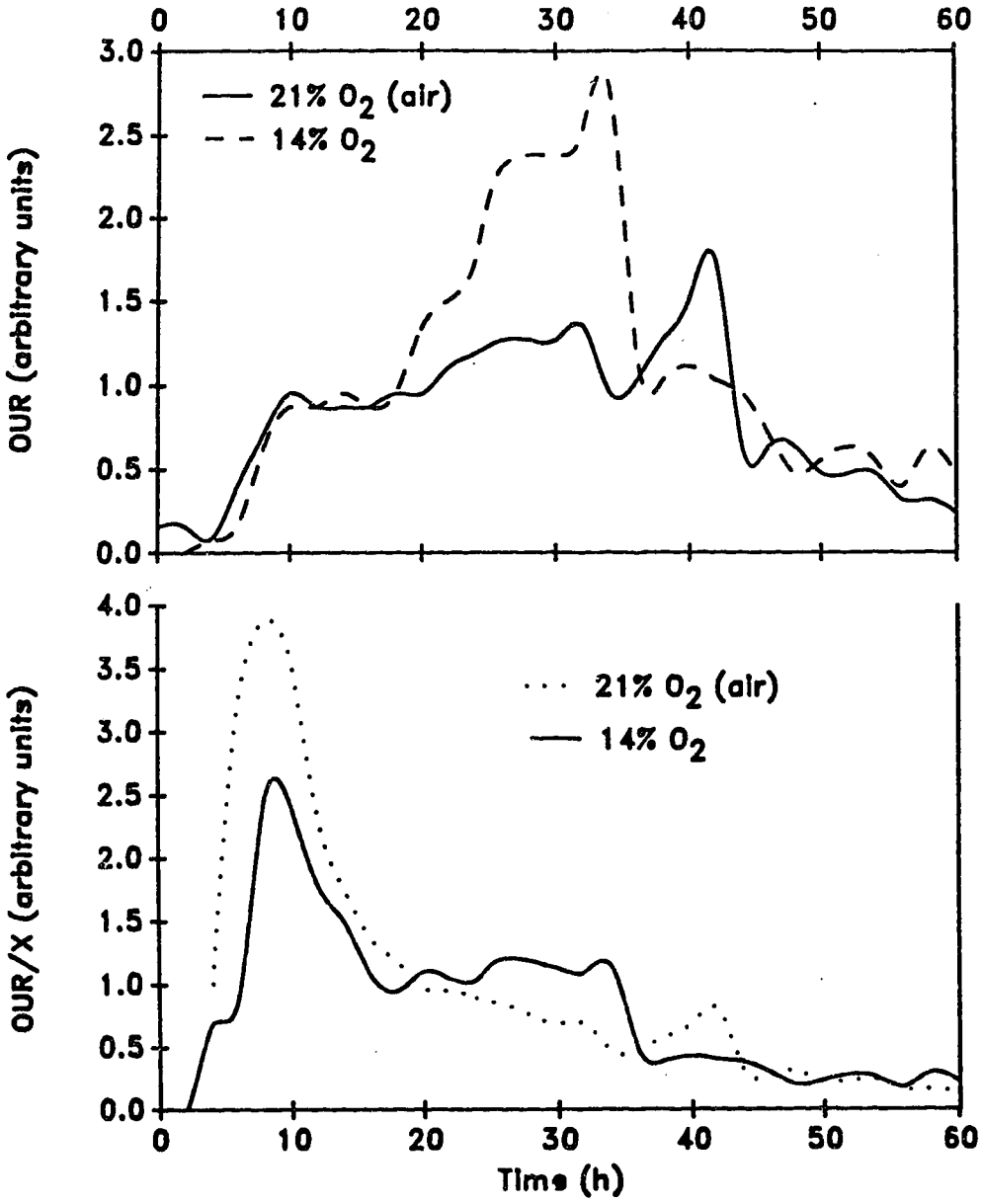

Figure 3.5 Time profiles of OUR and OUR/X.

Conclusions

The reduction in oxygen content at constant inlet gas flow to the fermentor increased asparagine utilization and protein synthesis. The lower specific yields of antibiotic suggest repression of the biosynthetic enzymes by reduced oxygen. The rapid loss of antibiotic after the substrate exhaustion may indicate derepression of hydrolytic enzymes at lower oxygen levels.

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CHAPTER 4

EFFECT OF DISSOLVED OXYGEN CONTROL ON GROWTH AND ANTIBIOTIC PRODUCTION IN *STREPTOMYCES CLAVULIGERUS* FERMENTATIONS

Introduction

Dissolved oxygen (DO) is known to be an important parameter in industrially important antibiotic fermentations. The critical dissolved oxygen for mycelial fermentations, such as *Streptomyces niveus*, can be as high as 50 % (Steel and Maxon, 1966). In penicillin production from *Penicillium chrysogenum*, irreversible damage by oxygen limitation has been noted (Vardar 1983, Vardar and Lilly, 1982).

Increased levels of dissolved oxgyen have lead to enhanced penicillin production in *Penicillium chrysogenum* (Hersbach *et al.*, 1984), and cephalosporin C production by *Cephalosporium acremonium* (Hilgendorf *et al.*, 1987). In secondary metabolite production, the optimal values for parameters such as pH, temperature and DO may not be the same for growth and metabolite production. Flickinger and Perlman (1980) found a two to threefold increase in neomycin production by *Streptomyces fradiae* when DO was maintained above 0.05 atm, by oxygen enrichment. The authors also found that addition of oxygen in pulses resulted in suppression of respiration (oxygen uptake rate), compared to continuous changes in the flow of oxygen. This suggests that the method used for DO control may affect growth and antibiotic production. During growth of *S. clavuligerus* on complex media, control of DO at 50 % and 100 % saturation increased the rate of specific cephamycin C production two–fold and three–fold, compared to the experiments without DO control (Rollins *et al.*, 1988). In a further study (Rollins *et al.*, 1990), the specific activities of two enzymes in the antibiotic biosynthetic pathway, deacetoxycephalosporin C synthase (DAOCS) and isopenicillin N synthase (IPNS)

were found to increase 2.3 fold and 1.3 fold respectively, when the DO was controlled at 100 % saturation throughout the fermentations. These previous studies indicate that DO level plays an important role in regulating the activities of the biosynthetic enzymes and thus affects antibiotic production. However, it is not clear if the effect of DO levels is related to the phase of the fermentation. Also, in the previous studies using DO control with *S. clavuligerus* (Rollins *et al.*, 1988, 1990), flow rates of O_2, and N_2 were regulated manually to control DO. As a result fluctuations in DO levels about the set point were observed. For example in one experiment, the DO level during the initial growth phase dropped from 100 % to 50 % over a period of 25 hours, and during the rest of the experiment varied between 30 % and 60 %. Furthermore, the monitoring of DO was not continuous. Tighter control on DO levels using computer monitoring and feedback control may be required to study the effects of different DO levels on growth and product synthesis. Since DO control by agitation, aeration, or pure oxygen requires some additional expenditure, it would be useful to determine the phase of fermentation during which DO control at saturation would be most effective. To our knowledge, no studies have been reported on the effect of control of DO at different levels during the growth and production phases of an antibiotic fermentation.

Several techniques have been used to control DO in fermentations. The use of agitation speed has been popular (e.g. Clark *et al.*, 1985). In this type of control, the DO reading is compared to the set point and the proportional–integral (PI) controller sends the control signal to the agitation motor which adjusts the impeller speed. The aeration rate to the fermentor has also been used as a control variable for DO control (Williams *et al.*, 1986). In these methods, the gas–liquid mass transfer coefficient (K_la) is altered to change the value of DO. This also changes the K_la for other gases such as CO_2, which is often undesirable. In case of DO control using agitation speed, and to a certain extent gas flow (Nishikawa *et al.*, 1977) the

shear environment in the fermentor is changed. As a result, the effect of controlled DO on growth cannot be studied independent of other factors, especially for shear sensitive organisms. Chen *et al.* (1985) used a system for DO control at low values under constant shear conditions. The DO reading from the probe is sent to a pneumatic controller which operates a control valve. The control valve regulates the flow rate of air to the reactor, and a makeup gas (1% CO_2 and N_2) is also fed to the reactor to maintain a constant total inlet gas flow rate. This system was further modified in a recent study (Smith *et al.*, 1990a) to allow the simultaneous control of DO and dissolved carbon dioxide for plant cell culture.

This study investigates the effect of constant controlled DO levels and stepwise control of DO levels during trophophase and idiophase on growth and antibiotic production in *Streptomyces clavuligerus*. A recent study with *Penicillium chrysogenum* found that higher stirrer speeds and greater frequency of circulation of mycelia through the high shear zone results in greater hyphal damage and lower rate of penicillin synthesis (Smith *et al.*, 1990b). The effect of shear on *Streptomyces* is not known. In order to maintain the shear and mass transfer characteristics constant, DO was controlled using an air/O_2 or N_2/O_2 stream as required, keeping the total gas flow rate constant. A PI control system was first developed to achieve this control.

Materials and Methods

Microorganism and Medium

The organism used in this study was *Streptomyces clavuligerus* NRRL 3585. The composition of the medium used for shake flask and fermentor cultures was as follows: tryptone, 17 g/L; soy peptone, 3 g/L; NaCl, 5 g/L; K_2HPO_4,1.25 g/L; soluble starch, 10 g/L; 1mL of trace elements solution previously described (Aharonowitz and Demain, 1978) per 1 liter of medium. MOPS buffer at 20 g/L was used for the shake flask cultures. The pH was adjusted to 6.8 with HCL prior to autoclaving.

Equipment

The studies were conducted using a 2 L New Brunswick Multigen fermentor, equipped for computer monitoring and control. A schematic diagram of the fermentor data acquisition and control system is given in Figure D.1. Sensors for pH (Phoenix autoclavable double–reference electrode), temperature (Omega), and DO (Ingold autoclavable DO probe) were connected to a microcomputer via an OPTO–22 data–acquisition system. The pH was controlled by an Omega PHCN–36 pH/ORP controller. A Tylan Mass flow controller system coupled to the computer via the OPTO–22 system was used to regulate the flow of gas to the fermentor. The on–line measurements were recorded every 15 minutes. A Dycor (CBE 200) mass spectrometer was used to for on–line analyses of the fermentor off–gas for O_2, N_2 and CO_2.

Culture and Assays

The inoculum (50 mL) was grown for 24 h at 28 °C and 250 rpm in a 250 mL shake flask. A 2 % inoculum was used to innoculate 1.3 L of media in the 2 L fermentor. The initial pH of all media was adjusted to 6.8, and controlled during the

fermentation between 6.6 and 6.9 with 1N NaOH or 4N HCl. The fermentor was agitated at 400 rpm and aerated with 1.5 litres per minute of air. The foam was controlled using 2 % antifoam SAG 471 (Union Carbide).

Samples were taken at intervals for biomass, protein, and antibiotic analyses. Biomass concentrations were measured by absorbance at 660 nm using a Spectronic 21 spectrophotometer. The dry cell weights were measured by filtering a known volume of sample and drying it to a constant weight at 100° C. The absorbance was linearly related to dry cell weight in the range 1.0 to 10 g/L. Sample volumes of 1.5 mL were centrifuged, and the supernatant used for antibiotic assays. The cells were washed, resuspended in water, sonicated and used for protein analysis. Production of β–lactam antibiotics (penicillin N and cephamycin C) were quantified by a bioassay using *E.coli* ESS as the indicator organism (Brana *et al.*, 1985). The amount of cephamycin C was determined by inclusion of penicillinase in the bioassay agar. The standard curve was developed using cephalosporin C. Proteins were determined using the dye–binding assay (Sedmack *et al.*, 1977) on the supernatant obtained from the centrifugation of sonicated cells. Bovine Serum Albumin (BSA) was used as the protein standard.

DO *control system*

Gas mixtures, air/O_2 or O_2/N_2 were used for control of DO at 100 % and 50 % saturation respectively. DO levels were monitored by the computer every 5 seconds. The DO values were fed to an exponential filter of the form,

$$\hat{y}_i(k) = y_i(k) + 0.5\,(\hat{y}_i(k-1) - y_i(k)) \qquad (4.1)$$

where $y_i(k)$ is the raw DO measurement, and $\hat{y}_i(k)$ is the filtered measurement at the k th instant.

Control calculations were performed using a PI algorithm on the filtered DO values every 60 seconds. The control signals were sent to the Tylan mass flow controllers,

to regulate the amount of O_2, N_2 and air, keeping the total gas flow to the fermentor constant at 1.5 lpm. For control of DO at 100%, air was supplemented with pure oxygen when required according to the PI algorithm. Air and nitrogen streams were used for DO control at 50 %.

The velocity or incremental form of the PI algorithm was used,

$$\Delta u(k) = K_C (T_S K_I + 1) e(k) - K_C e(k-1) \quad (4.2)$$

$$u(k) = u(k-1) + \Delta u(k) \quad (4.3)$$

where $e(k) = (y_{sp}(k) - \bar{y}_i(k))$, the error (% DO) between the setpoint and the filtered DO measurement, u(k) is the control signal lpm of O_2, $\Delta u(k)$ is the required increment in the control signal, T_s is the sample time in seconds, K_C and K_I are the proportional and integral constants of the controller.

The process reaction curve method or the Cohen–Coon method was used initially to tune the controller (Stephanopoulos, 1984). Step changes were introduced in the fraction of oxygen in the gas used for aerating the fermentor, and the transient response of DO monitored at various stages of the fermentation. The use of PI constants, $K_C$and K_I obtained by this method resulted in oscillatory behaviour during a fermentation with DO control at 100 %. In this study the objective of this controller was to regulate the DO at constant values (e.g. 100 %, 50 %). The controller constants obtained by the Cohen–Coon method were detuned to $K_C = 0.001$ and $K_I = 0.02\ s^{-1}$. These PI constants resulted in good control for DO set points at both 100 % and 50 %.

Four kinds of experiments were performed : (1) Control experiments without DO control. (2) DO control at 100% saturation throughout the experiment. (3) DO control at 100% during the growth phase, no control later (4) DO control at 100% during the growth phase, 50% later.

Results and Discussion

DO control

Figure 4.1 shows the DO profiles for the different modes of control. Setpoint regulation at 100% and 50% was achieved using a single set of detuned controller constants as described previously. For the experiment with a set point change from 100 to 50% at the end of the growth phase, the controller was able to adapt without overshoot. The set point was changed from 100 to 50 % when the carbon dioxide in the fermentor off gases dropped after reaching a maximum. The response to the setpoint change was slow and 20 min were required to reach the new setpoint. This slow response was, however, acceptable for the present study and also for most microbial systems. In a batch fermentation, the biomass increases and so does the oxygen uptake rate (OUR) and the total demand for oxygen. From classical control theory, we would need to update the PI constants to compensate for the changes in OUR. Adaptive controller schemes have been recently proposed (Cardello and San, 1988., Smith *et al.*, 1990a) to use the on–line measurement of OUR to update the controller parameters for DO control. In the only experimental study in this regard, Smith *et al.* (1990a) compared a proportional controller with an adaptive controller and demonstrated the superiority of the adaptive scheme in terms of reduced oscillations. The setpoint regulation of DO obtained in this study suggests that a PI scheme with constants corresponding to low OUR is adequate for most microbial processes. The model based controller proposed by Smith *et al* (1990a) would be an alternative method, eliminating the need for tuning required in the PI scheme, and giving consistently good control based on a physical knowledge of the process.

Effect of DO on growth

Table 4.1 summarizes the effect of different modes of DO control on growth of *S. clavuligerus*. The specific growth rate, μ for the 50 % DO control experiment is

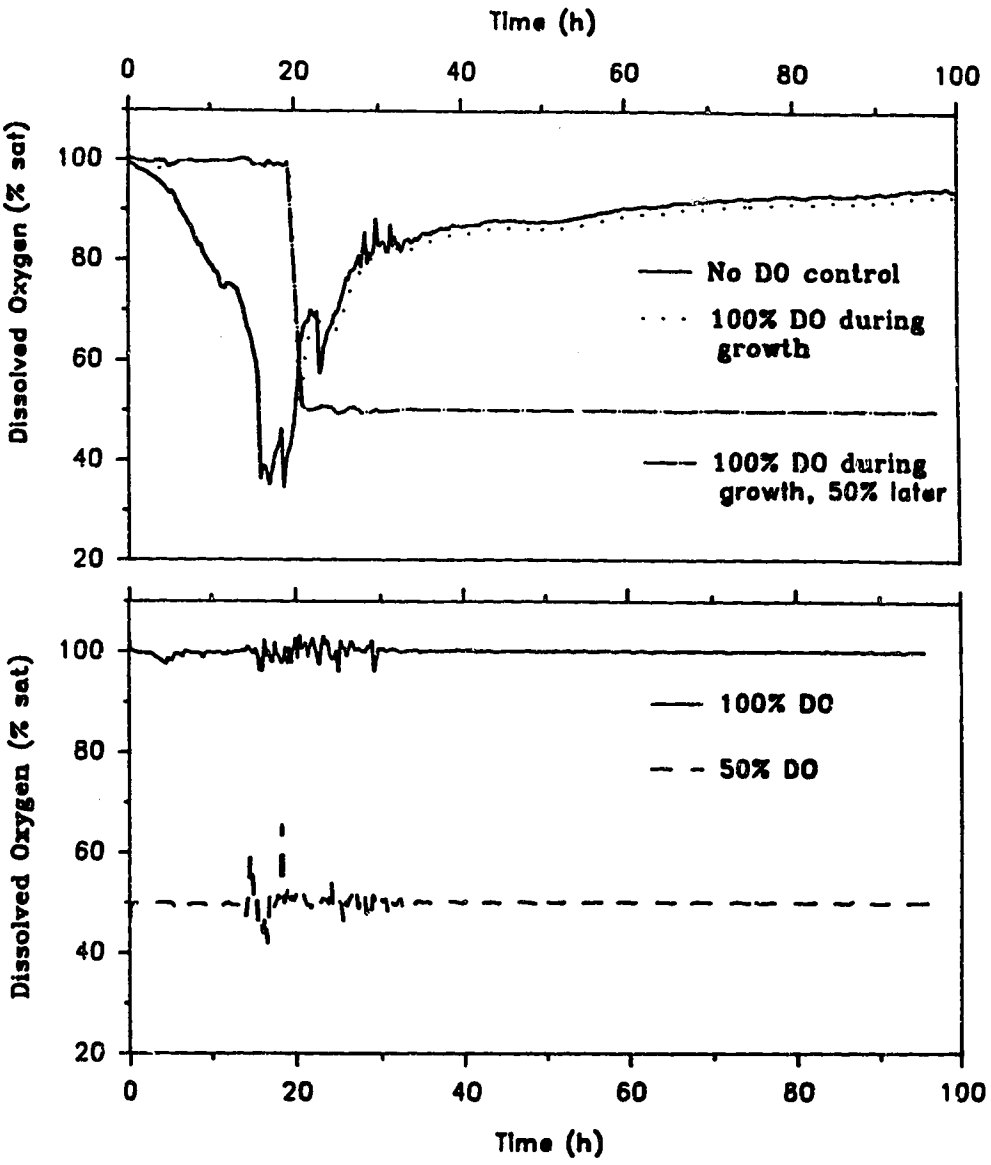

Figure 4.1 DO profiles during different modes of DO control.

Table 4.1 Growth parameters for experiments with different modes of DO control.

DO condition	Specific growth rate μ (h^{-1})	Maximum biomass (g DCW/L)	Protein fraction (g Protein/g DCW)
no control	0.21	8.65	0.232
100 % sat	0.193	8.95	0.253
50% sat	0.131	8.39	0.301
100% sat during growth	0.24	8.87	0.261
100% sat during growth 50% later	0.25	8.9	0.255

about 37 % lower than the experiment with no DO control. These lower specific growth rates for 50 % DO control experiments were reproducible, but the effect on the final amount of biomass (dry cell weight) was a small 3 % reduction compared to the experiment without DO control. The specific growth rates and final biomass levels for the experiments with DO control at 100 % were not significantly different from the experiment without DO control. The mean protein fractions in the biomass ranged from 0.23 to 0.3 in the different experiments and were fairly constant during the course of an experiment. A t–test showed no significant differences between the mean protein fractions at 95 % confidence intervals. These results here are in agreement with previous work on *S. clavuligerus* by Rollins *et al.* (1988) who reported no significant differences in growth based on proteins for experiments with DO control. The data on growth parameters (Figure 4.1) show that oxygen was growth limiting in the experiment with DO control at 50 %.

Effect of DO control on antibiotic production

Figure 4.2 compares the cephamycin C yields obtained in the different DO control experiments. In the experiment without DO control, cephamycin production started at 40 h and reached a final concentration of 70 mg/L at 70 h. The 50 % DO control experiment resulted in final cephamycin C concentrations of only 45 mg/L. Rollins *et al.* (1988) found an increase in cephamycin C production when DO was not allowed to drop below 50 % after this level was attained by consumption due to growth. However, in their base case experiment the DO level dropped to zero for about 10 hours during the growth phase. In the present study, the DO levels dropped only to 35 % for about 5 hours, and were in the range 100 – 50 % during most of the growth phase in the experiments with no DO control (Figure 4.2). Hence, control of DO at 50 % during the initial growth phase was actually

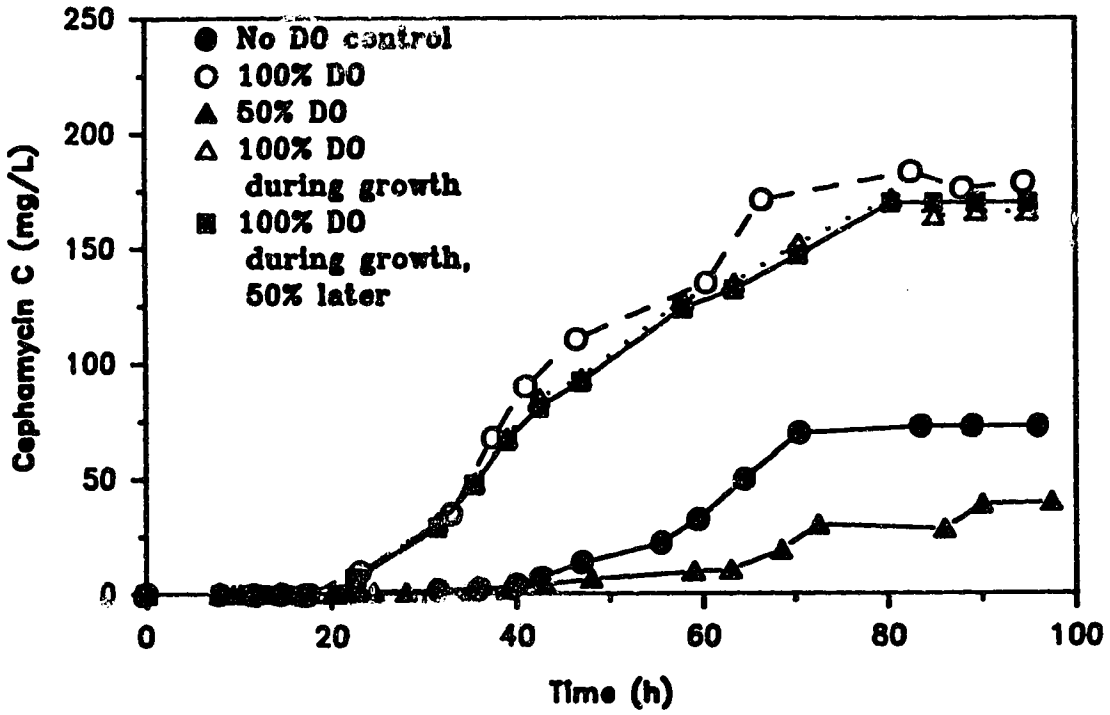

Figure 4.2 Cephamycin C profiles for the DO control experiments.

Table 4.2 Specific rates of Cephamycin C production during different modes of DO control

DO condition	Specific rate of Ceph C production (mg/g DCW/h)	Specific rate of Ceph C production (mg/g protein/h)	Ceph C / Total antibiotics
no control	0.26	1.14	0.55
100 % sat	0.64	2.98	0.87
50% sat	0.25	0.83	0.62
100% sat during growth	0.60	2.31	0.81
100% sat during growth 50% later	0.59	2.35	0.76

detrimental to the culture. The specific rate of cephamycin C production was comparable for the control and the 50 % DO control experiments (Table 4.2). For the experiments with DO control at 100%, specific rate of cephamycin C production, and the final concentrations were consistently better than the control experiments (Figure 4.2 and Table 4.2). Cephamycin C production commenced earlier (25 h), and within experimental error the final cephamycin C concentration for the different modes of DO control at 100% were identical at around 175 mg/L, a 2.4 fold increase relative to the control case. The final cephamycin C yields were thus independent of the DO level during the phase of production, when it was controlled at 100% during the growth phase. The specific rate of synthesis, however was greater for the experiment with DO control at 100% throughout the experiment by about 22% relative to the experiments where DO control was suspended after the growth phase or maintained at 50% during the non–growth phase (Table 4.2). The ratio of cephamycin C to the total antibiotics was also greater for the experiments with DO control at saturation relative to the experiments without DO control or DO control at 50%. Previous studies on the effect of dissolved oxygen on antibiotic production have indicated an improvement in production, even when oxygen enrichment was restricted to short periods of active respiration. Flickinger and Perlman (1980) showed significant increases neomycin production by *Streptomyces fradiae* as a result of oxygen enrichment during the peak growth phase (19–34 h). In secondary metabolism, the biosynthetic machinery responsible for production is set up during the growth phase. In *S. clavuligerus*, the β–lactam synthetases IPNS (isopenicillin N synthetase) and DAOCS (deacetoxycephalosporin synthetase) are produced during the phase of rapid growth (Rollins *et al.*, 1990). The enzyme IPNS is reponsible for synthesis of penicillin N and DAOCS converts penicillin N to cephamycin C. When DO was controlled at 100% saturation, the authors observed 1.3 fold and 2.3 fold

increases in the specific activities of IPNS and DAOCS respectively. Since the specific activity of DAOCS was higher relative to IPNS, this resulted in an accumulation of penicillin N and an increase in the ratio of cephamycin C to total antibiotics and the final cephamycin C concentrations. The actual mechanism of derepression of IPNS and DAOCS by high DO levels is unknown. Our observations are consistent with this previous study.

Maintaining DO levels at saturation during the growth phase appears sufficient to achieve high final concentrations of cephamycin C. Table 4.3 compares the effect of O_2 use on cephamycin C production for the experiments where respiration data were available. The gain in cephamycin C per unit pure oxygen used was the highest for the experiment where DO was controlled at 100% during the growth phase only. When pure oxygen was used, additional error from the mass flow controller is introduced in the calculation of oxygen consumption rates. Due to the lag time between the flow measurements and the gas analysis from the mass spectrometer, cumulative values for oxygen and carbon dioxide were used to calculate the consumption and production respectively. Although the oxygen consumption values for the experiments with DO control are not accurate, the total oxygen consumption was higher in the experiments where DO was maintained at 100%. This is consistent with higher CO_2 production in these experiments. Flickinger and Perlman (1980) observed similar trends in respiration data obtained during DO control experiments with *S. fradiae*. Yield coefficients of cephamycin C per unit oxygen consumed were lower for the experiments with DO control at 100%, compared to the control experiment.

Table 4.3 Yield of Cephamycin C and respiration characteristics for different modes of DO control.

DO condition	Pure O_2 used (moles/L)	Ceph C gain/O_2 used (mg/g)	O_2 consumed (moles/L)	CO_2 produced (moles/L)	Yield of Ceph C (mg/g O_2)
No control	0	0	0.248	0.187	9.2
100% sat	17.1	0.193	3.7	0.517	1.51
100% sat during growth	5.2	0.589	2.95	0.38	1.81

Cephamycin C production could possibly be improved relative to the production without DO control by maintaining DO above a certain intermediate level. Flickinger and Perlman (1980) were able to obtain a 55 % increase in neomycin production by control of DO above 0.05 atm. For implementation on the large scale, DO control at intermediate levels would also reduce the total oxygen consumption and hence the cost due to pure oxygen. Increasing the agitation speed to a level where the detrimental effects of shear could be avoided, would also reduce the amount of pure oxygen required for DO control.

Conclusions

The use of a PI control scheme was adequate to regulate the DO level using pure oxygen, to study the effect of DO on growth and antibiotic produc on. DO control at 50 % throughout the experiment r··ulted in lower specific gro· h rates and cephamycin C yields compared to experiments without DO ntrol. Maintaining DO at 100% saturation during the growth phase only was adequate to effect a significant 2.4 fold increase in the final cephamycin C yield, compared to experiments without DO control.

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CHAPTER 5

EXPERIMENTAL SIMULATION OF THE DISSOLVED OXYGEN ENVIRONMENT IN LARGE FERMENTORS USING A MONTE CARLO METHOD[1]

Introduction

Production–scale bioreactors range from 0.02–5 m^3 for animal cells and up to 300 m^3 for antibiotics and yeast production. As cells circulate in these agitated vessels, their local environment is primarily mixed and replenished near the impellers, and then left largely unmixed as the cells circulate into the peripheral volume. During the residency time in these poorly mixed zones, the cells can alter the local operating conditions. The cells in a large vessel, therefore, undergo fluctuations in local conditions which are dictated by the interaction between imperfect mixing and cell metabolism (Einsele *et al.*, 1978). Such fluctuations in the immediate environment of the cells can account for the difficulty in scaling up the production of many microbial cultures (Oosterhuis, 1984). Supply of oxygen in aerobic fermentations is always a concern because of its low solubility in water, and hence actively respiring cells would deplete the available oxygen in a relatively short time away from the oxygenated zone (Bryant, 1977; Oosterhuis *et al.*, 1985). This effect is particularly acute in viscous mycelial fermentations, where the transport of oxygen into the liquid is limited even in the vicinity of the impellers (Bailey and Ollis, 1986). Anaerobic conditions, created by local oxygen consumption, would inhibit both growth and synthesis of sensitive biosynthetic enzymes.

Fed–batch fermentation is commonly used for production of secondary metabolites. The biomass can be provided with the appropriate nutrients and

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[1]A version of this study has been accepted for publication as part of a paper in *Can. J. Chem. Eng.* (Namdev, *et al.*, 1990).

precursors at each stage of the fermentation, and fed–batch fermentation technology also offers operational benefits. For example, continuous addition of fresh medium can prolong production of antibiotic from *Cephalosporium acremonium*, and reduce catabolite repression (Trilli *et al.*, 1977; Matsumura *et al.*, 1981). In the production of yeast, the glucose is fed continuously to minimize the Crabtree effect. As fluid elements circulate in a large fermentor, the substrate will be mixed in at the impellers, and be depleted as the elements circulate through the vessel (Bajpai and Reuss, 1982). Occasionally the cells would be exposed to much higher concentrations as they pass the zone where the substrate is added (Fowler and Dunlop, 1989). A significant complication is the routine use of inexpensive complex media in industrial fermentation, which supply multiple carbon and nitrogen sources (Whitaker, 1980). In industrial bioreactors the local supply of both oxygen and substrates, therefore, can be influenced by mixing characteristics during fermentation.

A common method for characterizing circulation in stirred vessels is the mixing time, defined as the time for a pulse of tracer to mix with the bulk fluid (Nagata, 1975). Mixing times can be relatively long in production systems; as high as 104 s in a 160 m^3 fermentor (Charles, 1978). The circulation time distribution (CTD) is an alternative method of characterizing circulation within a stirred vessel, and is measured by determining the probability for each possible time interval that a fluid element takes to return to a fixed reference position, which is usually selected as the impeller region. The CTD for water in typical fermentors follows a log–normal distribution, with a mean time and a variance (Bryant, 1977; Middleton, 1979; Mann *et al.*, 1981; Mukataka *et al.*, 1981; Ruess, 1982; van Barneveldt *et al.*, 1987). This log–normal distribution is consistent with meandering flow paths away from the impeller region (Mann *et al.*, 1981). The mean circulation time increases with the size of the fermentor, up to 20 or 30 seconds for a 100–120

m^3 volume (Oosterhuis, 1984; Bryant, 1977; Middleton, 1979). With a mean time of 20 s, times from 10 s to 80 s would be observed for each pass around the fermentor. The breadth of the distribution of circulation times tends to increase with the viscosity of liquid, so that viscous fermentation media can give a bimodal distribution, indicating the development of stagnant zones in the vessel (Funahashi *et al.*, 1987).

One of the challenges for understanding and defining scale–up of microbial cultures, intended for processes carried out in large–scale stirred vessels, is to replicate the characteristics of this circulation distribution which have the largest impact on the cells. Such a scale–up method would use simple, small–scale equipment so that scale–up can be examined cheaply and systematically on the lab bench before moving to the production plant.

Several techniques have been proposed to investigate the effects of mixing and fluctuating local environment on growth and productivity of cells. Periodic oscillations in oxygen concentration and substrate concentration (Vardar and Lilly, 1982; Heinzle *et al.*, 1981; Heinzle *et al.*, 1985) have been proposed as methods to evaluate the effects of poor mixing in fermentors. Oxygen would be supplied every 20 s, for example, to mimic a production fermentor with a mean circulation time of 20 s. Circulation times in vessels are distributed over a range of values, however, and are not fixed at the single mean value. Furthermore, periodic oscillations in substrate concentration in chemostats can induce synchrony and shifts in metabolism which would not be characteristic of industrial fed–batch operation.

A two–fermentor system has been used to study the effects of dissolved oxygen fluctuations on *Gluconobacter oxydans* (Oosterhuis, 1984; Oosterhuis *et al.*, 1985) and *Saccharomyces cerevisiae* (Sweere *et al.*, 1988b), and fluctuations in glucose on *S. cerevisiae* (Sweere *et al.*, 1988c). By maintaining a high concentration in one tank, and low in another, continuous recirculation of fluid between the two

tanks can approximate circulation modes in a large fermentor. The two–tank approach is suggested by mass transfer studies which indicate that most micromixing occurs in a small zone near the impeller, with segregated flow in the remaining volume (Bajpai and Reuss, 1982; Wilhelm *et al.*, 1966). This two–tank experimental method suffers from several limitations. First, two fermentors must be kept in uncontaminated operation, and the flow between the two cannot be measured directly so that tracer methods must be used (Oosterhuis *et al.*, 1985). Second, the lowest concentration in the second vessel is dependent on both the activity of the culture and the rate of recirculation between the two tanks. Furthermore, the cells are exposed to only two concentrations rather than a continuous range. The third limitation is that the circulation time distribution is exponential (for the residence-time distribution for two tanks in series) rather than following a log–normal distribution. Hence the cells do not experience the same fluctuations that they would in a large vessel. The method cannot simulate the bimodal circulation observed for high viscosity systems (Funahashi *et al.*, 1987). Clearly, an alternative and more flexible method for studying mixing interactions is required.

This study presents a systematic method for designing small–scale experiments, in a single fermentor, to simulate the interaction between mixing and growth which is characteristic of large–scale production fermentors. The use of a Monte Carlo method to control the inputs to a small fermentor exposes the growing culture to representative fluctuations in local concentration. Experimental results are given for Monte Carlo cycling of air in batch and fed–batch cultures of *S. cerevisiae*. These experiments were conducted using a complex medium to better represent industrial fermentations with multiple carbon and nitrogen sources (Whitaker, 1980).

Theory

Bajpai and Reuss (1982) proposed a method for simulating the interaction between mixing and growth, following the mixing model of Manning *et al.* (1965). In this system, the agitated tank was split into a micromixing zone, a small portion of the tank volume in the vicinity of the impeller where all fluid elements were combined, and a macromixing zone (the remaining volume of the tank) where the elements were segregated. The time of passage through the macromixing zone and back to the micromixing zone was described by the following log–normal probability distribution:

$$f(t_c) = (1/\sqrt{2\pi}\ \sigma_l t_c)\ exp[-(ln\ t_c - \mu_l)^2/2\sigma_l^2)] \tag{5.1}$$

$$\bar{t}_c = exp\left[\mu_l + (\sigma_l^2/2)\right] \tag{5.2}$$

$$\sigma_\theta^2 = \left[\sigma^2/\bar{t}_c^2\right] = exp\ (\sigma_l^2) - 1 \tag{5.3}$$

where μ_l and σ_l are the mean and deviation of the normally distributed variable y ($\equiv ln\ t_c$). The distribution was divided into discrete elements, with $\bar{t}_c$ and σ obtained from the literature, and the model was used to simulate the effect of circulation on growth of yeast in a chemostat. This method is limited by the availability of valid models for growth and product formation, therefore a direct experimental method is required.

A simple approach to scale–up is to manipulate a small fermentor to directly simulate large–scale fluctuations. Hence, we consider the circulation of a single cell from a Lagrangian reference frame and make the small fermentor a representative fluid element in a much larger system. A cycle of the representative fluid element

through the fermenter is as follows: the fluid element starts in the micromixing zone, where the concentration of air or nutrient is replenished. The remainder of the circulation time (t_c) is in the macromixing zone, where the cells in the fluid element are left to depend on the local concentration of oxygen or nutrient. For example, by turning the air supply on and off, the cells in a small fermenter are alternately exposed to aeration and respiration–driven oxygen depletion, analogous to a large–scale system. The Monte Carlo method provides a straightforward technique for controlling the on–off cycles to mimic the circulation–time distribution in a much larger vessel: a random number can be used to select the circulation time (t_c) from the log–normal probability distribution as follows:

$$t_c = exp(R_n \sigma_l + \mu_l) \tag{5.4}$$

In practice $\bar{t}_c$ and σ are calculated from experimental data, then μ_l and σ_l are obtained by solving equations (5.2) and (5.3). The disadvantage of equation (5.4) is that very long cycle times are calculated, which may not be observed experimentally. Alternately, equation (5.1) can be discretized into n elements of equal probability, each with a corresponding circulation time which is log–normally distributed as in Figure 5.1. A uniform random number, R_u, is then used to select the current value of t_c. In the present study the distribution was divided into discrete elements, and $\bar{t}_c$ and σ were obtained from the literature. Note that any probability distribution could be studied by this discrete method. Over a large number of cycles the cells experience the log-normal distribution of circulation times. Because the circulation times are short with respect to the growth rate of the cells, the outcome of an experiment is not sensitive to the order of the cycles. Over the course of their growth the cells would experience thousands of these random cycles, so that no one circulation time would be dominant, unlike the case of periodic oscillations.

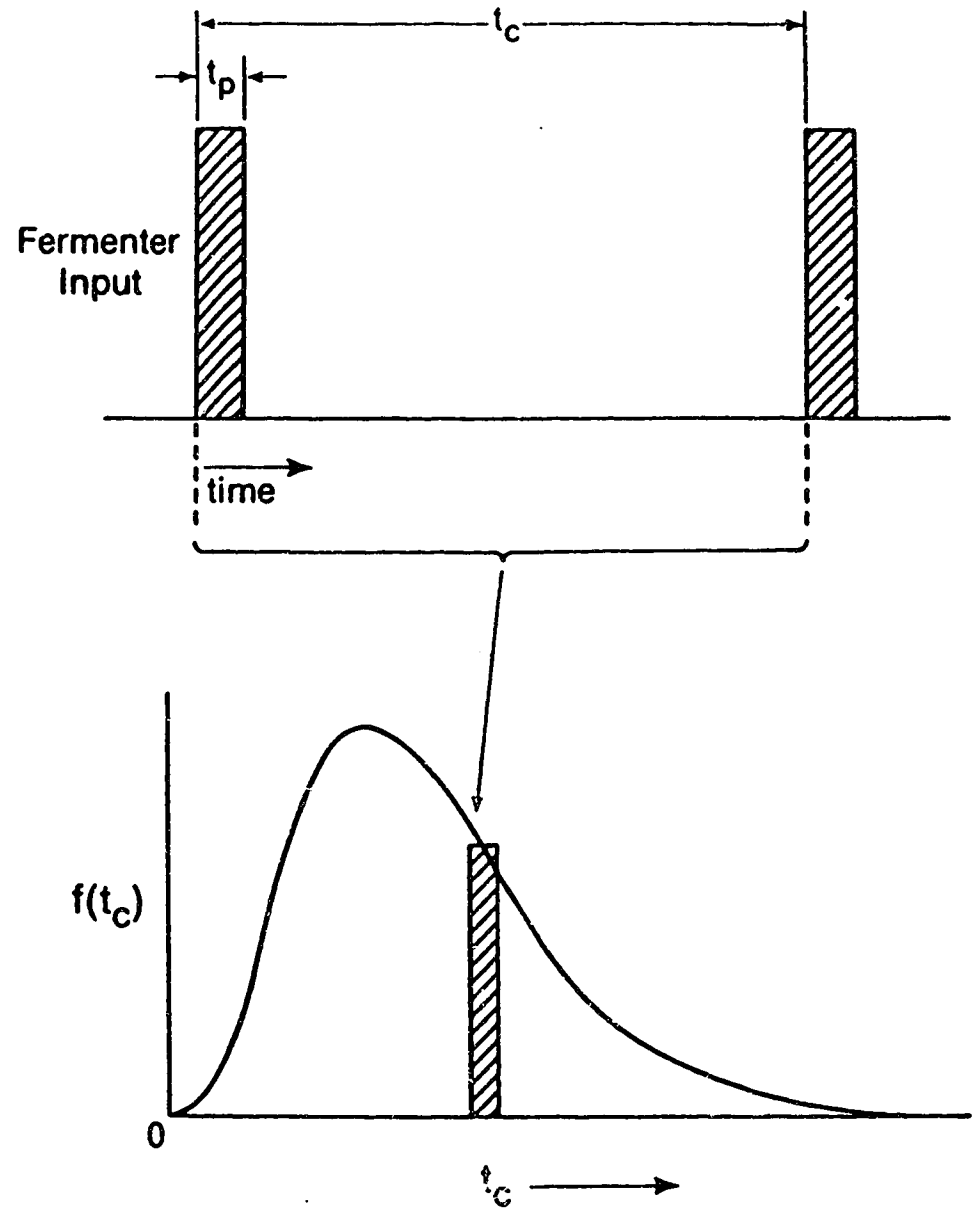

Figure 5.1 Schematic diagram of a single cycle in the Monte Carlo experiment. Air or nutrient is supplied for time t_p, then the feed is turned off for the remainder of the circulation time t_c. The values of t_c for successive periods are randomly selected from the log–normal distribution, $f(t_c)$.

Materials and Methods

Microorganism and Medium

The organism used in this study was *S. cerevisiae* NCYC 1018. The organism was maintained on a YD medium (1 % yeast extract, 2 % dextrose and 2 % Difco peptone, 2 % Bacto agar) slant. The composition of the medium used for shake flask and fermentor culture was as follows: dextrose, 10 g/L; yeast extract, 3 g/L; malt extract, 3 g/L; peptone, 5 g/L (all Difco). The feed composition for fed–batch runs was as following: dextrose, 100 g/L; yeast extract, 30 g/L; malt extract, 30g/L; peptone, 50 g/L (all Difco). RO water was used for the preparation of medium.

Equipment

The studies on fluctuations in oxygen and substrate concentration were conducted using 0.002 m^3 (2 L) New Brunswick Multigen fermentors, equipped for computer monitoring and control. A schematic of the fermentor data acquisition and monitoring system in given in Figure D.1. Sensors for pH (Phoenix autoclavable double–reference electrode), amount of acid and base added, temperature (Omega), and dissolved oxygen (Ingold autoclavable DO probe) were connected to a microcomputer via an OPTO–22 data–acquisition system. The pH was controlled by an Omega PHCN–36 pH/ORP controller. The fermentor off–gas was analyzed for O_2, CO_2 and N_2 by a Perkin Elmer Mass Spectrometer. A Tylan Mass flow controller system and peristaltic pumps (Pharmacia model P1; 0.6–500 mL/h) were used to regulate the flows of gas and substrate to the fermentors.

Cultures and Assays

The inoculum (50mL) was grown to exponential phase for 24 h at 30°C and 250 rpm in a 250mL shake flask. The fermentation was carried out with 2% inoculum and initial content of 1 L at 30°C, 500 rpm, and air supply of 1 L/min.

The pH was continuously controlled between 4.5 and 4.8 by using 2N HCl and 2N NaOH. The foam was controlled using 2% antifoam SAG 471 (Union Carbide). All fed–batch experiments were fed the feed medium at a rate of 13.5 mL/h. Feeding was started when the carbon dioxide evolution rate (CER) was 5–5.1 mmol/L/h and the glucose level was close to zero, after about 11 h of growth. At this stage the culture was switching from aerobic fermentation on glucose to respiration on ethanol.

Samples were taken aseptically every 2 h in the batch and every 3 h in the fed–batch experiments. The biomass was measured by absorbance at 620 nm using a Spectronic 21 spectrophotometer. A linear correlation between dry weight and absorbance was obtained. The sample was filtered using 0.45μm Sartorius cellulose nitrate filters. Glucose in the supernatant was analyzed using a YSI (model 27) Glucose Analyzer. Ethanol and acetic acid was measured within 1% precision using Spectra Physics GC equipped with 80/120 carbopack B/6.6% carbowax 20M column and a Flame Ionization Detector.

Design of Cycling Experiments

Each cycle of a Monte Carlo experiment followed a set pattern, as illustrated by a pulse of air or nutrient in Figure 5.1. A schematic diagram of the actual implementation is given in Figure D.2. The pulse time (t_p) was constant in all cycles, with a value of approximately half of the shortest circulation time. A log–normal distribution was used with parameters $\bar{t}_c = 20$ s and $\sigma = 8.9$ s, which characterize the circulation time distribution typical of a large fermentor (Bajpai and Reuss, 1982). The area under the distribution curve was divided into 25 elements, each of equal probability and represented by a particular circulation time. After the input of each pulse, one of the 25 circulation times (8 s $< t_c <$ 44 s) was randomly selected as the next total cycle time (t_c). The log–normal distribution was discretized in this manner to avoid extremely large values of t_c which would

occasionally result from equation (5.4). The batch and fed–batch experiments were conducted for 24 and 16 h (feeding time) respectively, encompassing a total of 3000 to 4500 cycles. The large number of cycles gave a good approximation to the log–normal distribution.

The effect of oxygen fluctuations was studied using batch and fed–batch experiments. Valves for air and /or nitrogen were switched on and off according to the Monte Carlo algorithm, running on a microcomputer. The pulse time was 5 s, corresponding to half of the slowest circulation time, to simulate passage through the impeller region. The chosen pulse time was also sufficient to re–aerate the fluid volume of 1 L. In one set of batch experiments, nitrogen was used during the off–time, to rapidly deplete the oxygen from the system. Two types of fed–batch experiments were conducted as controls: (i) continuous air supply of 1 L/min and (ii) periodic cycling with $t_p = 5$ s and $t_c = 20$ s.

Results and Discussion

The time profiles of dry cell weight (DCW) and ethanol for experiments with oxygen fluctuations in batch cultures using Monte Carlo air on/off and air/N_2 cycles are compared with the control experiment in Figure 5.2. A maximum DCW of 5 g/L was obtained for the batch control experiment as shown in Figure 5.2. This low yield of biomass, coupled with low oxygen uptake rates (OUR) (maximum 11 mmoles/L/h) ensured that the average dissolved oxygen (DO) remained above 30 % of saturation in the Monte Carlo experiments with air on/off (Figure 5.3). Monte Carlo experiments with air/N_2 cycles simulated a higher OUR, lowering the dissolved oxygen to 15% of saturation after 10 h. These experiments gave a small, but reproducible, 11 % decrease in DCW at the end of 24 h, and a 0.5 g/L increase in ethanol concentration (Figure 5.2). Oxygen supply limitations during the second phase of growth on ethanol probably resulted in lower ethanol consumption. The ethanol profiles were similar during the first phase of growth, up to about 10 h. Sweere *et al.* (1988b) observed similar effects in continuous cultures of baker's yeast subjected to periodic cycling of oxygen concentration (from air to N_2). The use of N_2 to induce fluctuations in dissolved oxygen also contributes to stripping of CO_2 from the system, and hence may not be representative of the DO fluctuations in a large–scale fermentor.

Fed–batch experiments were undertaken to study the effect of Monte Carlo oxygen fluctuations at higher DCW and OUR. Fluctuations in air supply according to the Monte Carlo method were compared with periodic cycling and continuous aeration. Figure 5.4 shows the DCW and ethanol profiles for the fed–batch experiments with oxygen fluctuations. The oxygen uptake rates (OUR) for the fed–batch experiments are shown in Figure 5.5. Table 5.1 lists key results from the repeated experiments, and shows that the results were quite reproducible. The biomass production in the Monte Carlo experiments was lower than the control

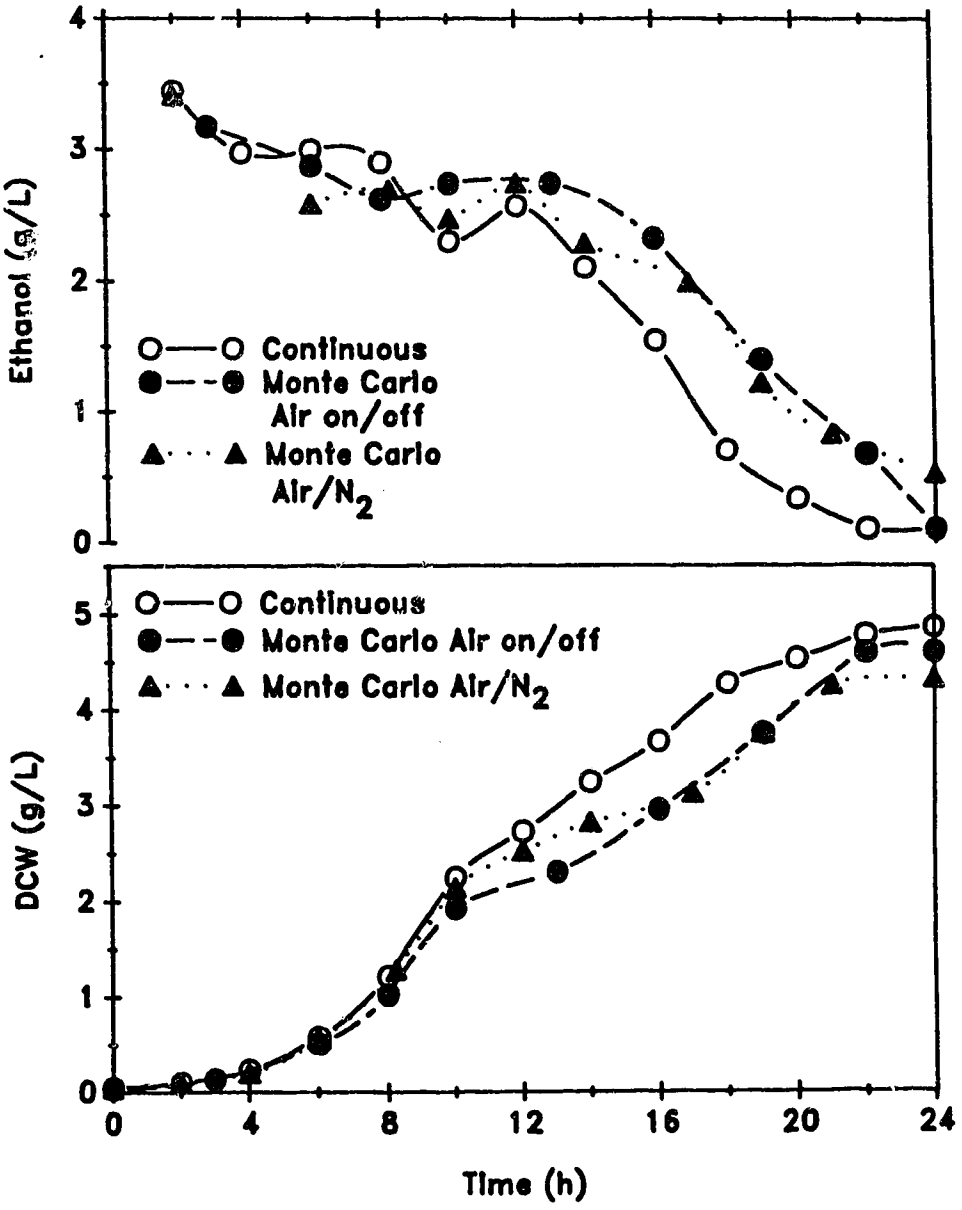

Figure 5.2 Yields of ethanol and biomass from batch fermentation of *S. cerevisiae* with different aeration strategies.

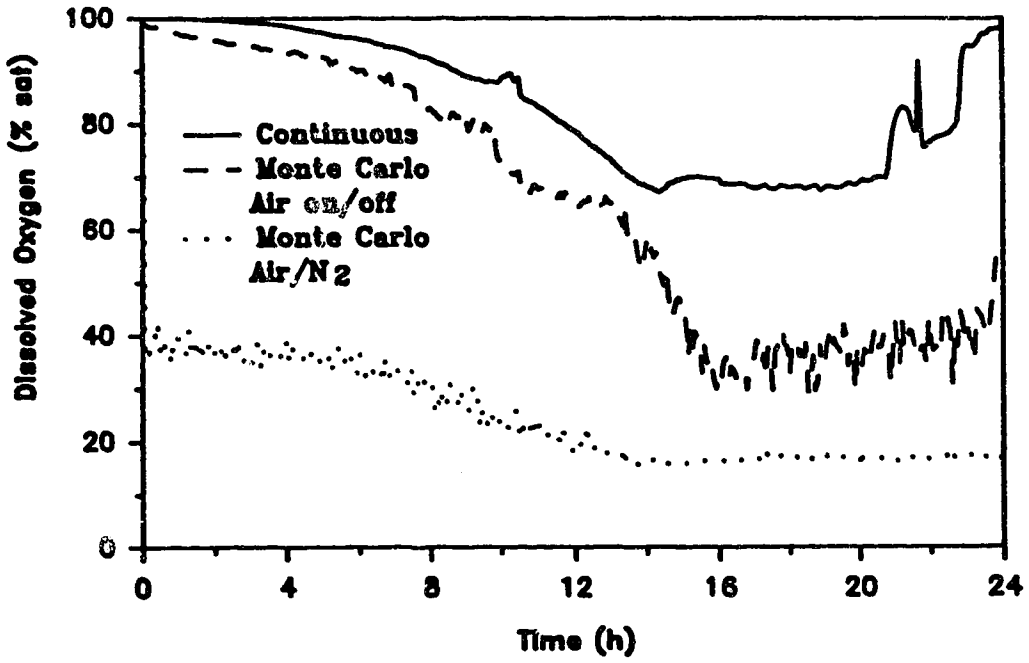

Figure 5.3 Dissolved oxygen profiles for batch fermentations of *S. cerevisiae*.

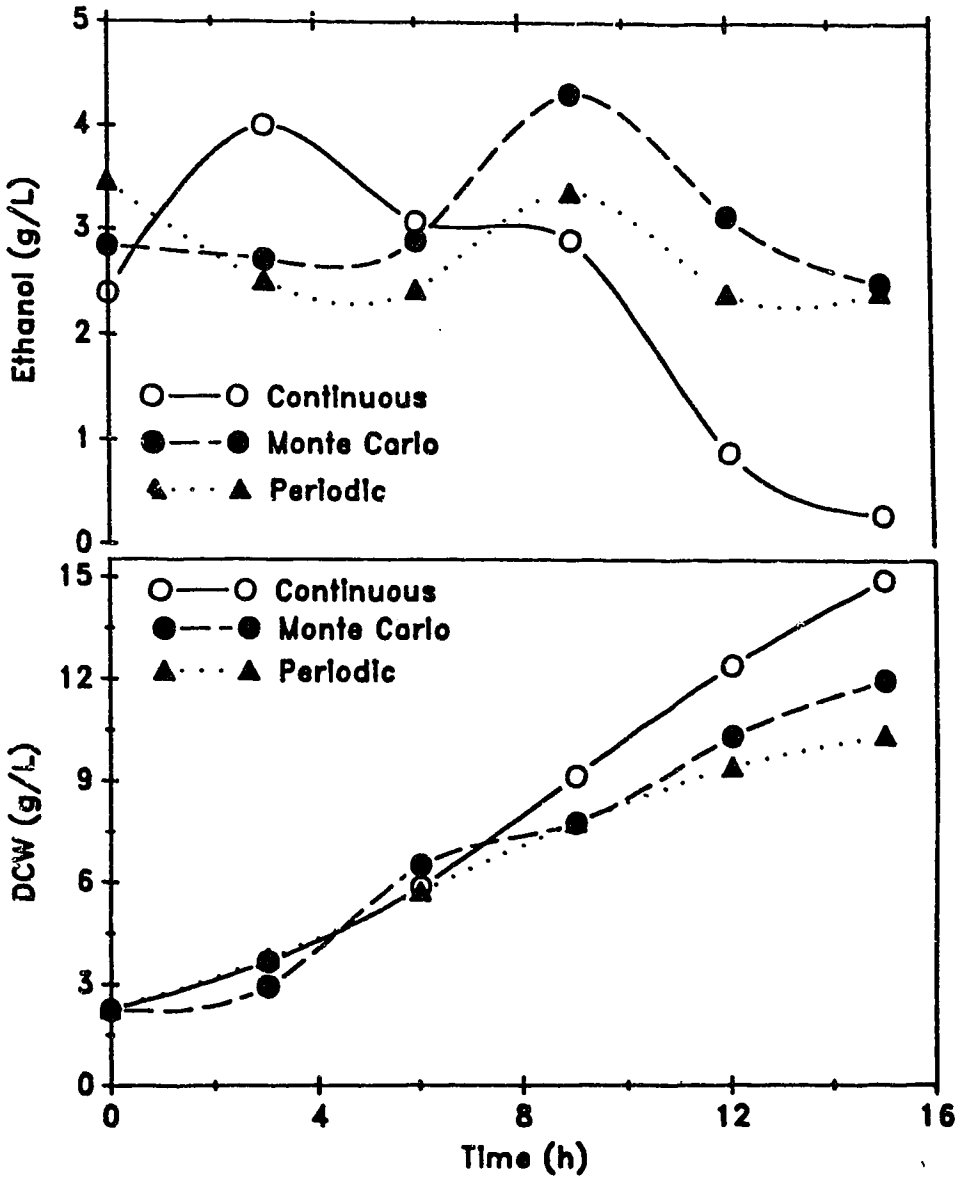

Figure 5.4 Yields of ethanol and biomass from fed–batch fermentation of *S. cerevisiae* with different aeration strategies.

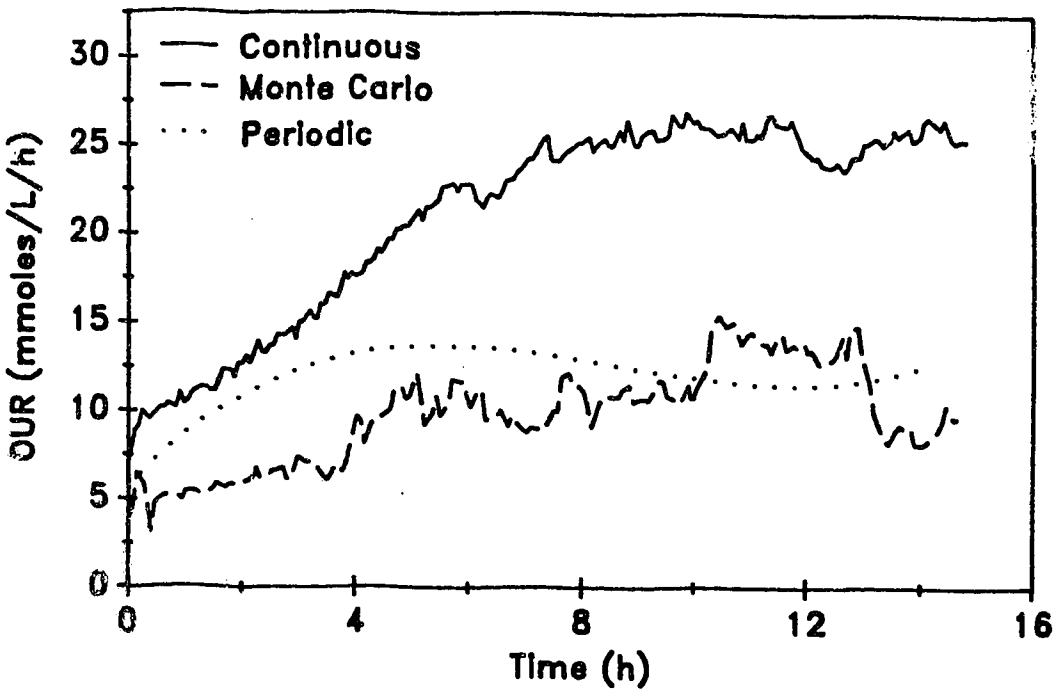

Figure 5.5 Oxygen Uptake Rate (OUR) during fed–batch fermentation of *S. cerevisiae*.

Table 5.1

Concentrations of Biomass, Ethanol, and Glucose From *S. cerevisiae* with Monte Carlo, Periodic, and Continuous Supply of Air

	Run	Terminal Biomass (g/L) (15 h)	Terminal Ethanol (g/L) (15 h)	Terminal Glucose (g/L) (15 h)
Continuous	1	14.58	0.23	0.35
	2	14.92	0.28	0.30
Monte Carlo	1	12.33	2.9	0.86
Air cycling	2	11.97	2.5	0.29
Periodic	1	10.36	2.42	0.26
Air cycling	2	10.6	2.53	0.30

experiment with continuous aeration by about 20 %, at the end of 15 h of feeding (Figure 5.4). A 30 % reduction in DCW was observed in the experiments with periodic cycling of air (5 s on, 15 s off). The differences in biomass levels appears after about 6 h of feeding time. The fluctuations in dissolved oxygen could not be recorded due to a lag time of about 10 – 15 s for the DO probe. The average dissolved oxygen values dropped close to 5 % at 4 h and 6 h for the periodic and Monte Carlo experiments respectively (Figure 5.6). These times indicated the onset of oxygen limitation, giving a reduction in biomass production after 6 h. In comparison, the dissolved oxygen level in the continuous aeration experiments was 40% at 4 h and 20% at 6 h. Earlier onset of oxygen limitation in the periodic experiment accounted for its final DCW, which was the lowest of the three experiments.

The mass transfer characteristics for each aeration procedure were evaluated by the dynamic method, measuring the dissolved oxygen in the fermenter after switching from nitrogen to air. The value of $k_l a$ was approximately 120 h^{-1} for continuous aeration, but the accuracy of this estimate is limited by the slow response of the dissolved oxygen probe. The periodic and Monte Carlo aeration experiments gave $k_l a$ of 51–56 h^{-1}, on a time–averaged basis. This result indicates that gas holdup during the off–cycle contributed to oxygen transfer, because the air was only on one fourth of the time (5 s on, 15 s off on average). These data suggest that the Monte Carlo method is affected by $k_l a$ and gas holdup in the small–scale fermenter.

The residual ethanol obtained near the end of the continuous aeration experiments was lower than in the two Monte Carlo or periodic experiments (Figure 5.4). The time profile of ethanol concentration was characterized by a maximum, which occurred at about 3 h for the continuous experiment, and at 9 h for the

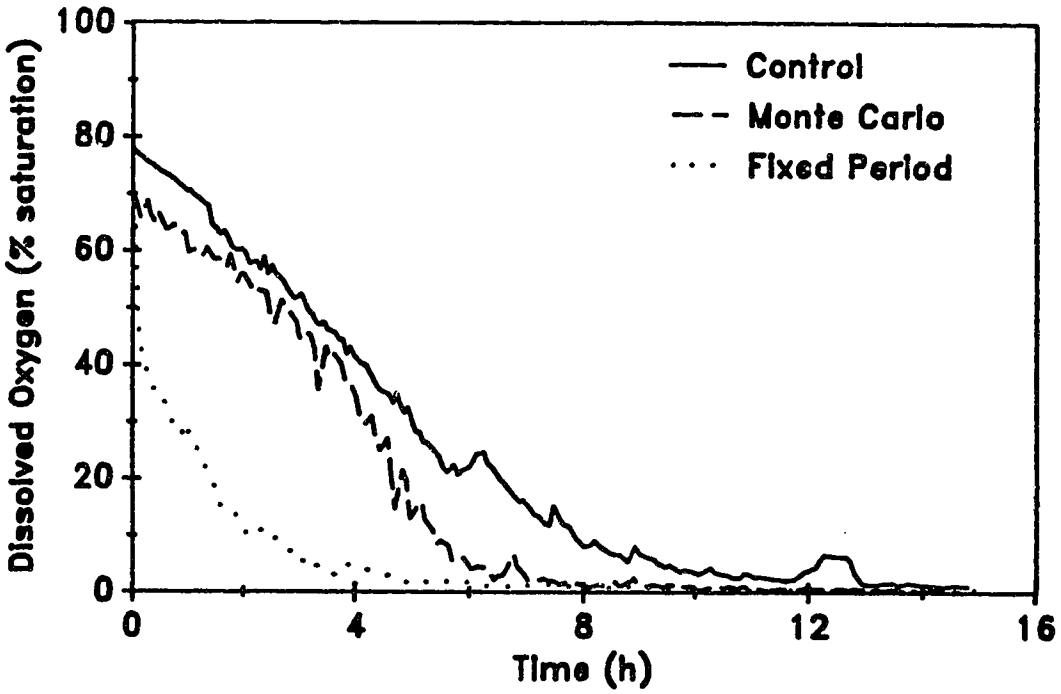

Figure 5.6 Dissolved oxygen profiles for fed–batch fermentations of *S. cerevisiae*.

Monte Carlo and periodic experiments. Simultaneous production and consumption of ethanol occurred during the course of the fed–batch run. Mass spectrometric analysis of the outlet gas from the fermenters showed that stripping of ethanol was insignificant. Oxygen limitation after 6 h in the experiments with fluctuations in oxygen supply may have lead to reduced ethanol consumption, or increased production as observed by Sweere *et al.* (1988b), resulting in higher accumulation of ethanol. Residual glucose concentrations in all fed–batch experiments were below 0.4 g/L and did not show any measurable differences as a function of time in the three types of experiments.

For the continuous and Monte Carlo experiments, OUR was calculated as a moving average over 4 minutes (Figure 5.5). In the periodic experiments, resonance between the frequencies of gas sampling at the mass spectrometer and the period of the inlet air flow resulted in apparent periodic behavior of OUR. The OUR from periodic experiments, therefore, was point averaged over 1.5 h. The higher OUR values for the continuous experiments were consistent with the higher DCW. The OUR values for the periodic experiments were higher than that for the Monte Carlo experiments up to 10 h of feeding time. After 10 h, the OUR was similar in Monte Carlo and periodic experiments, although DCW diverged. The carbon dioxide evolution rate (CER) profiles were similar to OUR.

Previous studies on the effects of oxygen fluctuations in baker's yeast have used the two fermentor system (Sweere *et al.*, 1988c) and periodic cycling in chemostat cultures (Sweere *et al.*, 1988a). The oxygen fluctuations decreased biomass production and increased ethanol accumulation. The results of the present study are in qualitative agreement with these previous studies. The differences in DCW values, and ethanol accumulation profiles between the Monte Carlo and periodic oxygen cycling in fed–batch experiments, for the same average circulation

time, indicate that the response of the culture to these two methods of cycling is different. Both the average circulation time and the distribution of circulation times, therefore, are important for the evaluation of culture response to fluctuations in DO for scale–up purposes. The results of periodic and two fermentor cycling experiments (*e.g.* Vardar and Lilly, 1982., Sweere et al., 1988a, 1988c) may not adequately describe the effects of oxygen fluctuations in a large fermentor.

An autocorrelation function could be used to characterize the signals for the three modes of aeration. In the large scale fermentor an autocorrelation function could be obtained for the circulation of a fluid element with respect to a reference point. Another approach to simulating the microenvironment of the cells in a large fermentor could be to expose the small fermentor to aeration signals which give similar autocorrelation as experienced on the large scale.

Conclusions

1. An experimental technique for evaluating the effects of circulation in stirred production bioreactors was developed based on a Monte Carlo method for duplicating the circulation time distribution on a small scale.
2. Monte Carlo cycling of air to *S. cerevisiae* cultures gave experimental results different from both periodic cycling and continuous supply.
3. The Monte Carlo experiments were quite reproducible because the randomly selected circulation times were much shorter than the length of the experiments.

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CHAPTER 6

EXPERIMENTAL SIMULATION OF DISSOLVED OXYGEN FLUCTUATIONS IN LARGE FERMENTORS : EFFECT ON *STREPTOMYCES CLAVULIGERUS*

Introduction

Aerobic fermentations for antibiotic production are generally carried out in large stirred fermentors of up to 300 m^3 in volume. Most antibiotic fermentations involve mycelial species such as *Penicillium, Cephalosporium,* and *Streptomyces.* These mycelial fermentations result in highly viscous, and often non–Newtonian broths (Van Suijdam and Metz 1981, Allen and Robinson 1990) resulting in bimodal circulation time distributions, and stagnant zones in the vessel (Funahashi *et al.*, 1987). Under such conditions, the transport of oxygen from the air bubbles to the mycelia via the bulk liquid is limited even in the vicinity of the impellers (Bailey, and Ollis, 1986), resulting in gradients in dissolved oxygen throughout the fermentor. The microorganisms in a large fermentor are thus subjected to a continuously changing dissolved oxygen in their microenvironment. The critical dissolved oxygen level for mycelial fermentations can be as high as 50 % of saturation (e.g. Steel and Maxon, 1966). Hence, exposure to low levels of dissolved oxygen could result in loss of productivity on scale–up. In order to effectively scale–up the operating parameters, and select process equipment, it would be useful to determine the effects of fluctuations in dissolved oxygen on growth and product formation.

Streptomyces clavuligerus produces four β – lactam compounds : two cephalosporins, penicillin N and clavulanic acid. An earlier study (Yegneswaran *et al.*, 1988) using defined media postulated that a reduction in oxygen content at constant aeration resulted in hydrolysis of antibiotics. During growth on complex media, control of dissolved oxygen at 50 % and 100 % saturation increased the rate

of specific cephamycin C production two–fold and three–fold, compared to the experiments without dissolved oxygen control (Rollins *et al.*, 1988). In a further study (Rollins *et al.*, 1990), the specific activities of two enzymes in the antibiotic biosynthetic pathway, deacetoxycephalosporin C synthase (DAOCS) and isopenicillin N synthase (IPNS) were found to increase 2.3 fold and 1.3 fold respectively, when the dissolved oxygen was controlled at 100 % saturation throughout the fermentations. These previous studies indicate that dissolved oxygen level plays an important role in regulating the activities of the biosynthetic enzymes and thus affects antibiotic production. This suggests that fluctuations in dissolved oxygen levels in large fermentors may, therefore, have an effect on antibiotic yields.

Few investigators have addressed the problem of studying the effects of fluctuations in dissolved oxygen, as experienced on a large scale, on antibiotic production. Vardar and Lilly (1982) observed a decrease in penicillin titres, when sinusoidal cycles with a period of 2 minutes were introduced on dissolved oxygen, between 37 % and 23 % of saturation. Actual circulation times in large fermentors are distributed over a range of values, and using a mean circulation time may not mimic the actual dissolved oxygen fluctuations. Larsson and Enfors (1985) showed that the inactivation of *Penicillium chrysogenum* (as measured by the oxygen uptake rate) by oxygen starvation followed first order kinetics. Oxygen starvation for a period of 1 min corresponded to a 4.7 % inhibition of the oxygen uptake capacity. The mixing time in large fermentors is in the range of minutes and is sufficient to damage the metabolic capacity of cells due to oxygen starvation. In a further study, Larsson and Enfors (1987) used a two compartment fermentor consisting of a well mixed aerated stirred vessel and an anaerobic plug flow section, to study the effect of dissolved oxygen gradients on the respiratory capacity of *Penicillium chrysogenum*. With 6 % of the total volume in the anaerobic section and 10 minutes of mean residence time, a reduction of respiration by 32 % was observed when the

total culture volume had passed through the anaerobic part. The effect on antibiotic production was not reported. In a two compartment system the cells are exposed to only two concentrations, or regular cycles in the case of stirred and plug flow sections, rather than a continuous range which exists in a poorly mixed large scale fermentor.

This study presents data on the effect of dissolved oxygen fluctuations on growth and antibiotic production in *Streptomyces clavuligerus*. A Monte Carlo method, as described in chapter 5, is used to mimic, in a small fermentor, the characteristic dissolved oxygen fluctuations experienced by the microorganisms in the large scale. Continuous and periodic aeration modes are used as controls.

Experimental Materials and Methods

Microorganism and Medium

The organism used in this study was *Streptomyces clavuligerus* NRRL 3585. The composition of the medium used for shake flask and fermentor cultures was as follows: tryptone, 17 g/L; soy peptone, 3 g/L; NaCl, 5 g/L; K_2HPO_4,1.25 g/L; soluble starch, 10 g/L; 1mL of trace elements solution previously described (Aharonowitz and Demain, 1978) per 1 liter of medium. MOPS buffer, 20 g/L was used for the shake flask cultures. The pH was adjusted to 6.8 with HCL prior to autoclaving.

Equipment

The dissolved oxygen fluctuation studies were conducted using a 2 L New Brunswick Multigen fermentor, equipped for computer monitoring and control. Sensors for pH (Phoenix autoclavable double–reference electrode), temperature (Omega), and dissolved oxygen (Ingold autoclavable DO probe) were connected to a microcomputer via an OPTO–22 data–acquisition system. A schematic of the fermentor data acquisition and control system is given in Figure D.1. The pH was controlled by an Omega PHCN–36 pH/ORP controller. A Tylan Mass flow controller system was used to regulate the flows of gas and substrate to the fermentor. The sampling interval for all on–line measurements were 15 min.

Culture and Assays

The inoculum (50 mL) was grown for 24 h at 28 °C and 250 rpm in a 250 mL shake flask. A 2 % inoculum was used to innoculate 1.3 L of media in the 2 L fermentor. The initial pH of all media was adjusted to 6.8, and controlled during the fermentation between 6.6 and 6.9 with 1N NaOH or 4N HCl. The fermentor was agitated at 400 rpm and aerated with 1.5 lpm of air. The foam was controlled using

2 % antifoam SAG 471 (Union Carbide).

Samples were taken at intervals for biomass, protein, and antibiotic analyses. Biomass concentrations were measured by absorbance at 660 nm using a Spectronic 21 spectrophotometer. The dry cell weights were measured by filtering a known volume of sample and drying it to a constant weight at 100° C. The absorbance was linearly related to dry cell weight in the range 1.0 to 10 g/L (Appendix A). Sample volumes of 1.5 mL were centrifuged, and the supernatant used for antibiotic assays. The cells were washed, resuspended in water, sonicated and used for protein analysis. Production of β–lactam antibiotics (penicillin N and cephamycin C) was quantified by a bioassay using *E.coli* ESS as the indicator organism (Brana *et al.*, 1985). The amount of cephamycin C was determined by inclusion of penicillinase in the bioassay agar. The standard curve was developed using cephalosporin C. Proteins were determined using the dye–binding assay (Sedmack *et al.*, 1977) on the supernatant obtained from the centrifugation of sonicated cells. Bovine Serum Albumin (BSA) was used as the protein standard.

Design of air cycling experiments

Two types of air cycling experiments were conducted : (i) Monte Carlo controlled aeration and (ii) periodic aeration. For Monte Carlo controlled aeration, a log–normal distribution was used with parameters $\bar{t}_c = 20$ s and $\sigma = 8.9$ s, which characterize the circulation time distribution typical of a large fermentor (Bajpai and Reuss, 1982). The area under the distribution curve was divided into 25 elements, each of equal probability and represented by a particular circulation time. The total cycle time was randomly selected from one of the 25 circulation times. The air was turned on for 5 s (t_p) during each cycle. The air flow to the fermentor was turned off during the rest of the cycle. Experiments were also conducted with periodic cycling of air with $t_p = 5$ s and $t_c = 20$ s.

Results and Discussion

Figure 6.1 shows the DO profiles for the three types of experiments. In the control experiments, the lowest DO during the period of peak demand was in the range 40 to 50 %. As described in chapter 5, the actual fluctuations in DO due to interruptions in air flow in the Monte Carlo and periodic aeration experiments could not be recorded due to the response characteristics of the DO probe. The DO levels in the Monte Carlo and periodic aeration experiments dropped to the range 0 to 10 %. The results were found to be reproducible by repeating the experiments atleast three times.

Figure 6.2 shows representative time profiles for dry cell weight for the three kinds of experiments. Each of the profiles are characterized by a prominent lysis phase after 40 h. As shown in Table 6.1, the specific growth rate (μ) is higher for the continuous aeration experiment by 15 to 20%. No significant difference was observed in the corresponding maximum dry cell weights. The average protein fraction was also not significantly different for the three types of experiments. Previous studies on the effects of DO cycling on antibiotic producing organisms have not reported any changes in biomass levels. Larsson and Enfors (1985) reported a reduction in the oxygen uptake rate, as a result of interruption in oxygen supply during trophophase. In chapter 4 we observed that control of DO level at 100% during the fermentation does not alter the growth characteristics of *S. clavuligerus*, but control of DO at 50 % throughout the experiment reduced the specific growth rate. The visual observation of broth samples under a microscope showed mostly filamentous growth with few clumps. Apparent viscosity measurements were made for some samples using a Brookfield viscometer. The maximum apparent viscosity observed was 150 cp, corresponding to a maximum DCW of about 8.5 g/L. The apparent viscosity measurements were constant at different shear rates, indicating that the broth was a Newtonian fluid.

Table 6.1 Growth parameters for experiments with different modes of aeration.

	Run	Specific growth rate μ (h^{-1})	Protein fraction (g Protein/g DCW)
Continuous	1	0.255	0.260
	2	0.23	0.257
	3	0.24	0.265
Monte Carlo	1	0.173	0.253
	2	0.182	0.262
	2	0.166	0.270
Periodic	1	0.195	0.266
	2	0.186	0.259
	3	0.191	0.272

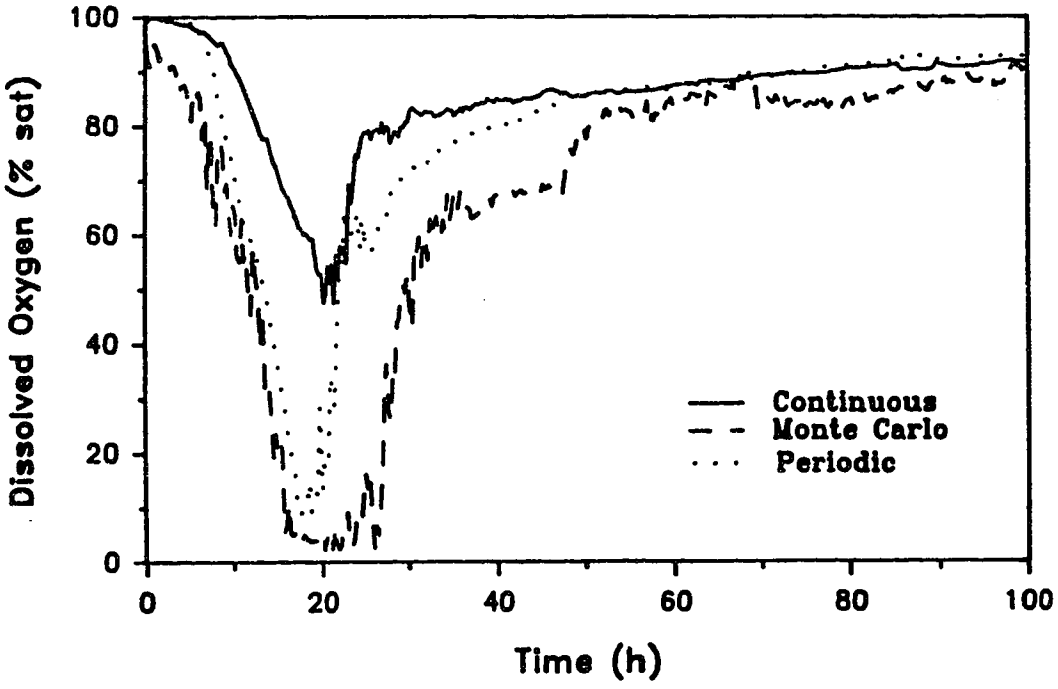

Figure 6.1 Dissolved oxygen profiles for *S. clavuligerus* fermentations with different aeration strategies.

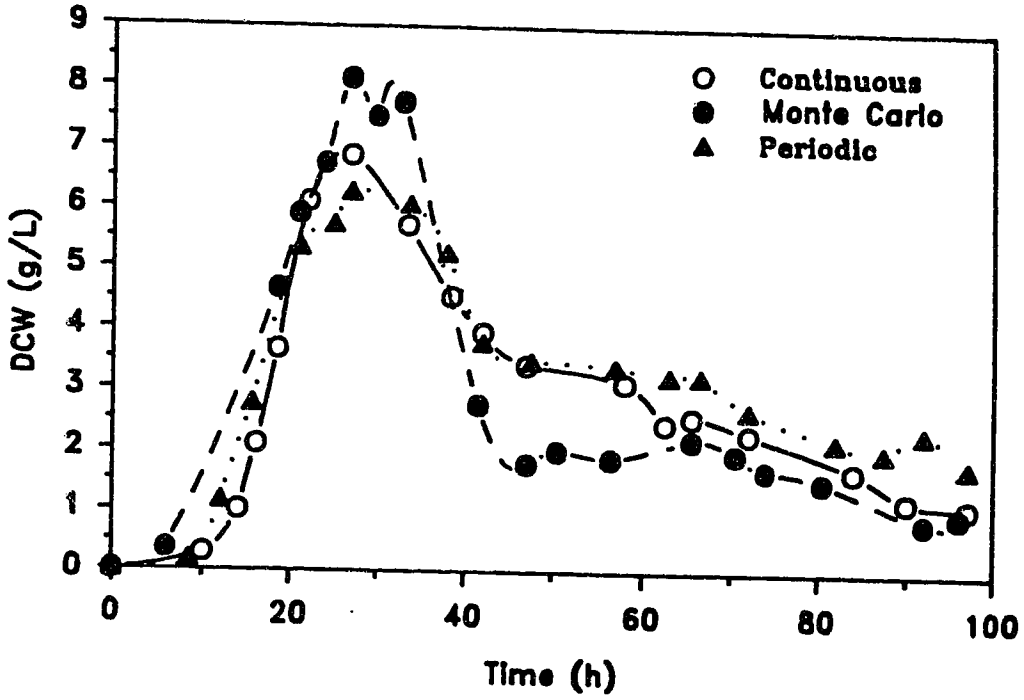

Figure 6.2 Biomass (DCW) profiles for *S. clavuligerus* fermentations with different aeration strategies.

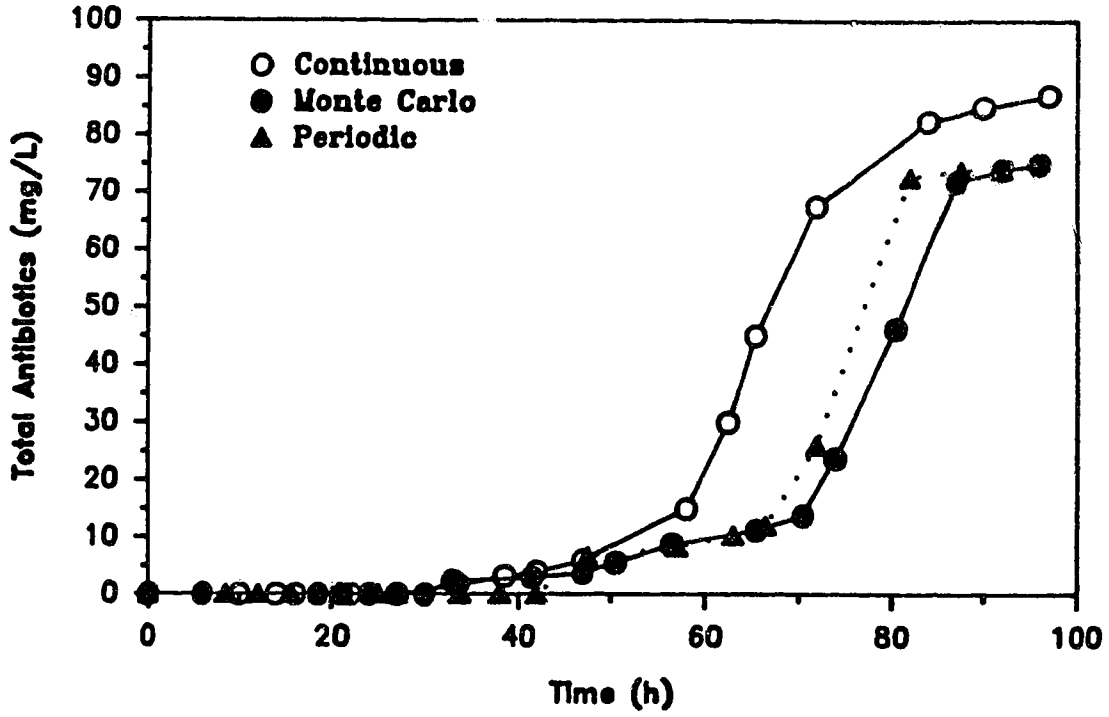

Figure 6.3 Profiles of total antibiotics for *S. clavuligerus* fermentations with different aeration strategies.

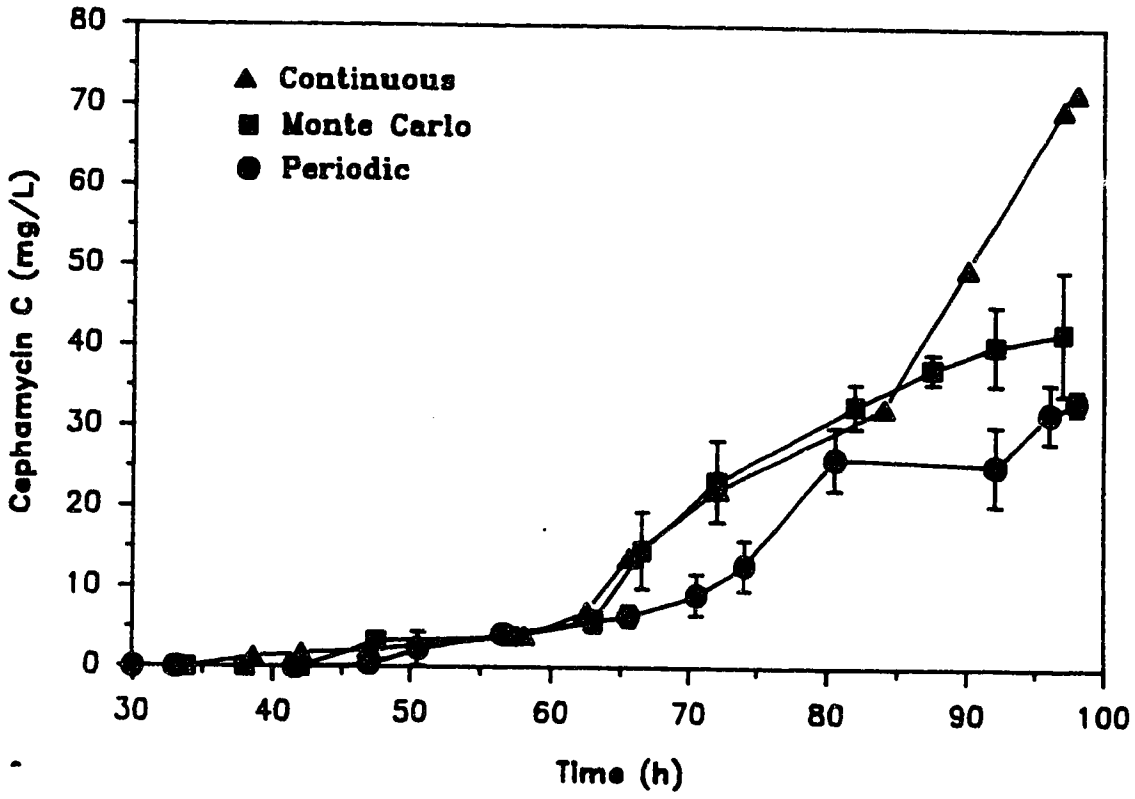

Figure 6.4 Cephamycin C profiles for *S. clavuligerus* fermentations with different aeration strategies

Figure 6.3 shows the time profiles for the total antibiotics (cephamycin C and penicillin N). In the experiment with continuous aeration, production begins at about 40 h and a final concentration of 90 mg/L was obtained. In the Monte Carlo and periodic aeration experiments, production begins at the same time as the continuous aeration experiment but at a lower rate until about 70 h. Final concentrations of 78, and 73 mg/L were obtained in the Monte Carlo and periodic aeration experiments respectively. Figure 6.4 shows the time profiles of cephamycin C production. The final cephamycin C concentrations for the Monte Carlo and periodic aeration experiments were 56 and 40 % lower than the continuous aeration experiment respectively. The Monte Carlo experiment showed lower cephamycin C yield than the periodic experiment (error bars at 95 % confidence intervals). The average specific rates of cephamycin C production were almost equivalent for the three cases (Table 6.2). The ratio of cephamycin C to total antibiotics was lower for the Monte Carlo and periodic experiments by 30 and 15 % respectively. Since the final total antibiotic concentrations were similar for the Monte Carlo and periodic experiments, an increase in cephamycin C represented improved conversion from penicillin N to cephamycin C in the periodic aeration experiments. In the repeated experiments, the cephamycin C concentration obtained from the Monte Carlo experiments was lower than the periodic experiments by about 15 % (Table 6.2).

In the Monte Carlo aeration experiments, the cycle time during which the aeration is interrupted could be as high as 44 seconds. During the active growth phase this could result in longer periods of oxygen starvation than during the periodic aeration experiments. As described in chapter 4, the enzymes IPNS and DAOCS were likely responsible for the effect of DO on biosynthesis of cephamycin C. The enzymes IPNS and DAOCS synthesize penicillin N and cephamycin C respectively. The data from this study are consistent with the enzyme levels

Table 6.2 Specific Cephamycin C production rates and final antibiotic levels for different modes of aeration.

	Run	Specific rate of Ceph C production (mg/g DCW/h)	Ceph C (mg/L)	Total antibiotics (mg/L)	Ceph C/ Total antibiotics
Continuous	1	0.297	75	90	0.83
	2	0.285	56	96	0.58
	3	0.279	55	85	0.65
Monte Carlo	1	0.295	31	78	0.4
	2	0.301	34	75	0.46
	3	0.280	40	80	0.50
Periodic	1	0.293	36	73	0.49
	2	0.281	41	75	0.55
	3	0.275	49	79	0.62

measured by Rollins *et al.* (1990). Reduced oxygen levels suppressed both total antibiotic yields and the fraction of cephamycin C, as would be expected if oxygen regulates both IPNS and DAOCS enzymes. The lower antibiotic levels observed during the Monte Carlo, as compared to the periodic aeration experiments, were consistent with the longer periods of oxygen deprivation.

Rollins *et al.* (1990) suggested that oxygen derepresses the synthesis of IPNS and DAOCS to enhance antibiotic formation. Their data, and the results of the present study, are consistent with the regulation of enzyme synthesis by DO levels, though the actual mechanism by which DO affects enzyme activities is not known. An alternate explanation to derepression is that the regulation of these enzyme activities by DO is the effect of promoters on synthesis. In a recent study, an oxygen sensitive promoter element (ORE) from *Vitreoscilla* was cloned into *E. coli* (Khosla and Bailey, 1989, Khosla *et al.*, 1990) and the expression of protein was induced at DO levels below 5 % air saturation. In *Streptomyces*, though the phenomenon of catabolite repression on antibiotic biosynthesis is well established, the mechanism for the effect of catabolite repression on enzyme activities has not been identified at the genetic level. The role of oxygen effects on the enzyme activities at the genetic level can only be resolved after dealing with the effect of catabolite repression. The present knowledge about *Streptomyces* genetics in relation to antibiotic biosynthesis indicates the presence of multiple linked promoters having heterogeneous sequences (Vining and Doull, 1989). It is possible that the some of these promoters are regulated by oxygen levels. The lower average DO levels during the growth phase in the Monte Carlo and periodic aeration experiments probably resulted in lower expression of IPNS and DAOCS, leading to lower total antibiotics and cephamycin C.

Conclusions

Cycling of aeration to the fermentor by the Monte Carlo and periodic methods resulted in no significant effect on the growth characteristics. In the Monte Carlo and periodic aeration experiments lower total antibiotics and cephamycin C concentrations were obtained compared to the continuous aeration experiments. The final cephamycin C levels were lower for the Monte Carlo aeration experiments compared to the periodic case, consistent with lower expression of key biosynthetic enzymes.

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CHAPTER 7

KINETICS OF CO_2 HYDRATION IN FERMENTORS : pH AND PRESSURE EFFECTS[1]

Introduction

The importance of CO_2 in microbial fermentations has been recognised, and the detrimental effects of elevated pCO_2 on growth and metabolism have been discussed in recent review articles (Ho *et al.*, 1987, Jones *et al.*, 1982). Control of CO_2 in the exit gas to below 20 %, by increasing the aeration rate was shown to prevent inhibition of yeast growth (Shimizu *et al.*, 1984). The CO_2 evolution rate (CER) has often been used for on–line estimation of cell growth (Mou and Cooney, 1983). The respiratory quotient (RQ), calculated as a ratio of CER to O_2 uptake rate (OUR), has been used as an estimate of the metabolic state of a culture during a fermentation. Control of RQ by substrate feeding in baker's yeast production has been used in order to enhance biomass production (Aiba, 1976).

Dissolved carbon dioxide has been found to be an important variable in antibiotic fermentations. In the production of antibiotics tetracycline and oleandomycin by *Streptomyces* sp., both antibiotic yield and respiratory rate was decreased by 40–50 % at 0.15–0.2 atm pCO_2 (Bylinkina *et al.*, 1973). Ho and Smith (1986), in experiments with *Penicillium chrysogenum*, observed a reduction in penicillin production and cell growth rates by 23 and 40 % respectively, when the fermentation was aerated with 12.6% CO_2 gas. The authors postulate a build up of H_2CO_3 and HCO_3^- within the cells, as part of the inhibitory effect. In chapter 3, we discussed the possibility of lower pCO_2 levels, derepressing the synthesis of

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[1]A version of this study was published earlier in *Biotechnol. Bioeng.* (Yegneswaran, *et al.*, 1990).

hydrolytic enzymes. In order to study the effects of pCO_2 levels in fermentations it is important to examine the examine the factors affecting the equilibrium of CO_2 in fermentors.

The dynamic equilibrium of CO_2 in aqueous fermentation systems is affected by pH and pressure. Fluctuations in fermentor pH could lead to changes in off–gas CO_2 concentrations during the transient approach to new steady states by shifting the bicarbonate concentration in solution. Pressure fluctuations in the head space of the fermentor exert a similar effect on off–gas CO_2 concentrations and hence also on quantities calculated from it such as respiratory quotient. Such temporary changes in CO_2 concentrations could be misinterpreted as changes in microbial metabolism, resulting in faulty control actions if a control strategy is based upon CO_2 evolution rate or RQ measurements.

The objective of this study is to quantify the kinetics of changes in gas phase CO_2 due to changes in medium pH and head space pressure. A mathematical model is developed and compared to experimental data to determine the factors which control the transients of CO_2 hydration in fermentors. Water and a standard yeast growth medium were used as model systems.

Experimental Materials and Methods

The experiments were carried out in a 2 L Multigen (New Brunswick Scientific) and a 20 L (Chemap) fermentor for the study of pH and pressure effects respectively. Three different fluids were used for the experiments: distilled water, a yeast growth medium containing 3 g/l Bacto yeast extract, 3 g/l Malt extract, 5 g/l Bacto peptone, and 10 g/l Bacto dextrose, and a fermentation broth containing non–growing yeast. The fermentation broth contained 3 g/L biomass, and was obtained after the termination of a batch fermentation using the above medium. A gas mixture containing 1.5 % (v/v) CO_2, and 1.5 % (v/v) O_2 in N_2 was passed through the fermentor contents to simulate a typical effluent gas composition. The aeration rates in the 2 L and 20 L fermentors were 0.77 and 0.6 vvm, respectively. pH changes in the range of 4 – 6 were obtained by changing the set point on an on–off pH controller (Omega), which added either 2N HCL or 2N NaOH. Head space pressure in the fermentor was manipulated in the range $1 - 2 \times 10^5$ N/m^2, using a back pressure valve. The 2 L fermentor (working volume 1.3 L) was agitated at 500 rpm and the 20 L fermentor (working volume 10 L) was agitated at 350 rpm. Fermentor pH, pressure, dissolved oxygen and temperature were continuously monitored every 20 s using a computer monitoring system. Temperature was kept constant at 30^0 C in all experiments. The off gases were analysed on–line for CO_2, O_2 and N_2 using a Perkin–Elmer Mass Spectrometer, with a sampling interval ranging from 1 min (pressure experiments) to 2 min (pH experiments).

Theory

Model for CO_2 hydration kinetics

Upon dissolution in water, CO_2 forms H_2CO_3 which rapidly dissociates to yield H^+ and HCO_3^-. The overall rate law can be written as :

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \qquad (7.1)$$

(slow) (fast)

with k_{CO_2} = 0.025 to 0.04 s^{-1} (25^0 C) and $k_{H_2CO_3}$ = 10 to 20 s^{-1} (20–25^0 C) (Stumm and Morgan, 1981).

The rate of hydration of CO_2 in a closed system is given by (Stumm and Morgan, 1981) :

$$\frac{d[CO_2]}{dt} = -k_{CO_2}[CO_2] + \frac{k_{H_2CO_3}}{K_{H_2CO_3}}[H^+][HCO_3^-] \qquad (7.2)$$

where, the dissociation equlibrium constant for carbonic acid, H_2CO_3 is $K_{H_2CO_3}$ = 2 x 10^{-4} M (Eigen and Hammes, 1963). In a fermentor the dynamics of dissolved CO_2, and bicarbonate in the media can be described by the following equations :

$$\frac{d[CO_2]}{dt} = -k_{CO_2}[CO_2] + \frac{k_{H_2CO_3}}{K_{H_2CO_3}}[H^+][HCO_3^-] + k_{l}a\{[CO_2]^* - [CO_2]\} \qquad (7.3)$$

$$[CO_2]^* = H P x_i \qquad (7.4)$$

$$\frac{d[HCO_3^-]}{dt} = k_{CO_2}[CO_2] - \frac{k_{H_2CO_3}}{K_{H_2CO_3}}[H^+][HCO_3^-] \qquad (7.5)$$

A material balance on CO_2 around the fermentor gives the following equation :

$$(Q\,x_i - Q\,x_o)\frac{P^o}{R\ T} = V_l\,k_la\,\{[CO_2]^* - [CO_2]\}$$

or

$$x_o = x_i - \frac{R\ T}{P^o Q} V_l\,k_la\,\{[CO_2]^* - [CO_2]\} \qquad (7.6)$$

Equations (7.3 – 7.6) constitute the model which can be used to describe the effects of pH and pressure on CO_2 hydration. The model can be used to predict departures of x_o from its steady state value due to shifts in pH and pressure.

Equations (7.3 – 7.6) were solved, using the IMSL routine DVERK, for measured step changes in pH and pressure. Values for rate, equilibrium constants and Henry's constant were obtained from Stumm and Morgan (1981):

$k_{CO_2} = 0.04\ s^{-1}$

$k_{H_2CO_3} = 20\ s^{-1}$

$K_{H_2CO_3} = 2 \times 10^{-4}\ M$

$H = 0.0316\ M\ atm^{-1}$

The mass transfer coefficient (k_la) was adjusted to fit the experimental data. The values of k_la used for the pH and pressure experiments were 100 h^{-1} in a 2 L fermentor and 25 h^{-1} in a 20 L fermentor respectively.

Time Constants of Model

The eigenvalues of the model equations were evaluated in order to determine the dominant time constants, and their sensitivity to k_la and pH. Equations (7.3–7.5) can be written in matrix notation as,

$$\frac{d\bar{Y}}{dt} = A\,\bar{Y} \tag{7.7}$$

where $\bar{Y}$ is a column vector of variables,

$$a_{11} = (-k_{CO_2} - k_1 a)$$

$$a_{12} = \frac{k_{H_2CO_3}\,[H^+]}{K_{H_2CO_3}}$$

$$a_{21} = k_{CO_2}$$

$$a_{22} = \frac{-k_{H_2CO_3}\,[H^+]}{K_{H_2CO_3}} \tag{7.8}$$

The eigenvalues of the system are eigenvalues of A evaluated at a reference or steady state,

$$|A - \lambda I|_{ss} = 0 \tag{7.9}$$

and the time constants of the model are given by,

$$t_i = -1 / \lambda_i \tag{7.10}$$

Results and Discussion

Figure 7.1 shows the effect of pH step changes in water on off–gas CO_2 concentrations. A step up in pH results in a downward spike in CO_2 concentration and vice versa. Changes in pH in the range 4 – 6 induce a significant change (± 13%) in off–gas CO_2. Fluctuations in pH in the narrow range 4.7 – 4.9 towards the end of the experiment result in considerable changes in CO_2 concentrations. The fluctuations in CO_2 concentrations were significant despite the small fermentor volume and the slow rate of gas sampling. Using a value of 100 h^{-1} for k_la in a 2 L fermentor, the model fitted the experimental data well for the period 0 to 220 minutes. At 220 minutes, narrow limits were set on the pH controller and high acid/base addition rates were used to give rapid fluctuations in pH. The sampling time of 20 seconds was too slow to accurately follow the true pH and hence did not accurately predict the fluctuations in CO_2.

Good agreement between experimental data and the model prediction was also obtained for growth medium and a yeast fermentation broth, in the same fermentor, using the same set of model parameters as for distilled water. Due to the low concentration of components in the growth medium no correction was applied to the Henry's constant (Ho *et al.*, 1987). The maximum and minimum CO_2 concentrations during some transients were not measured due to the slow sampling rate of the mass spectrometer (2 – 2.5 min). Shifts in pH affect the capacity of the liquid phase to retain CO_2 according to equations (3–5). Corresponding changes in $[CO_2]$ and $[HCO_3^-]$ are shown in Figure 7.2.

The change in CO_2 due to pH was very fast in this 1.3 L volume with k_la at 100 h^{-1}. In larger fermentors the response would be slower and transients in CO_2 would be of longer duration. Many industrial fermentations are carried out without pH control, and even where pH is controlled, the value shifts between certain limits due to mixing times of the order of a 1–2 minutes, and loose control. According to

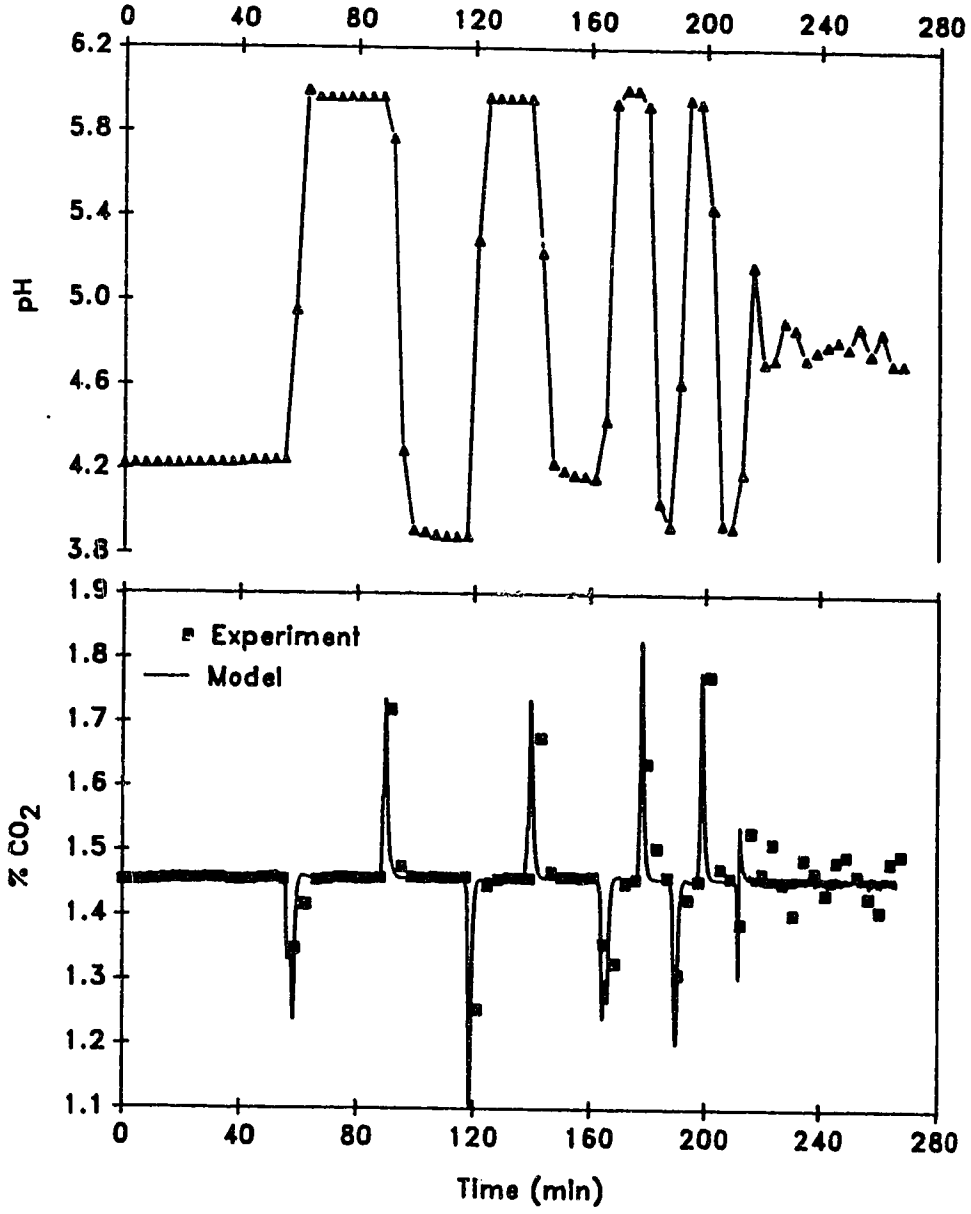

Figure 7.1 Effect of pH fluctuations in fermentor on $\%CO_2$ in off–gas, experimental data and model prediction.

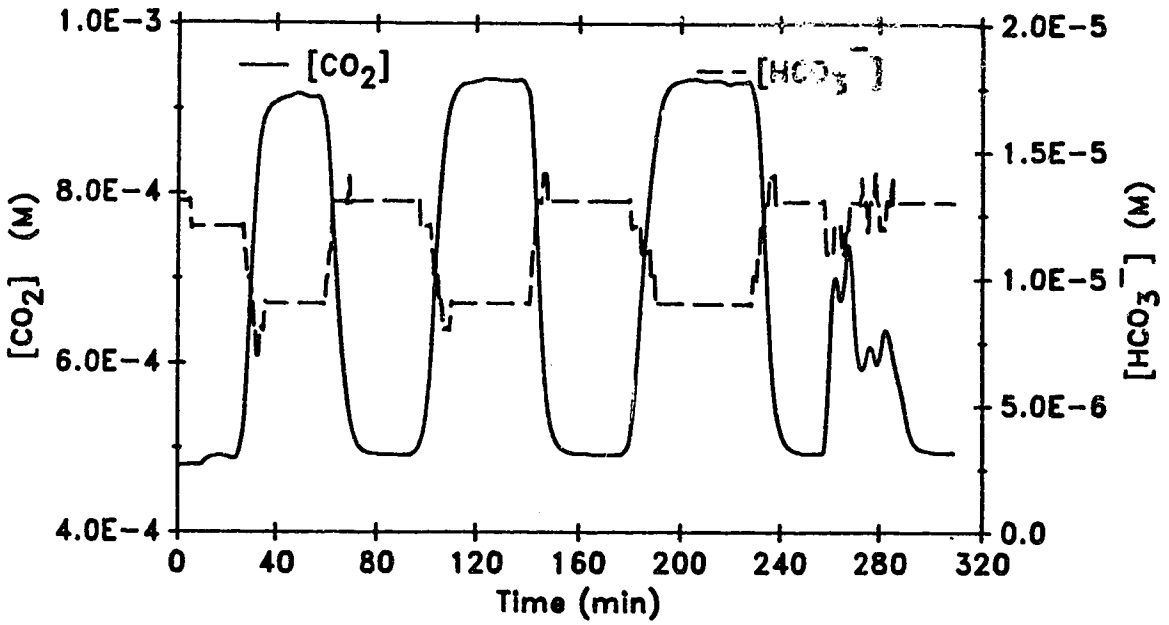

Figure 7.2 Model prediction of $[CO_2]$ and $[HCO_3^-]$ during pH fluctuations.

the model, a pH change of 0.2 units in a 1000 L working volume with k_la of 50 h^{-1} gives a maximum error of 56% in measured CO_2 concentration. As can be observed from equation 6 increases in V_l or k_la result in increases in magnitude of the error $(x_o - x_i)$. Lower k_la values would result in a lower magnitude of CO_2 fluctuations with longer transients, as explained later. In production of secondary metabolites, control of pH at different values during different stages of growth can be used to enhance product formation (Chu and Constantinides, 1987). Under such conditions it is important to distinguish changes in CER due to pH changes from actual changes in cellular metabolism.

Figure 7.3 shows the effect of shifts in fermentor headspace pressure on off–gas CO_2 concentrations. Pressure shifts in the range 1 – 2 atm result in fluctuations in CO_2 values up to ± 14%. An increase in pressure results in a down spike in CO_2 concentration and vice versa. Though the results shown here are for water, identical results were obtained with growth medium. Good agreement between experimental data and model predictions was obtained when a value of 25 h^{-1} was used for k_la in the 20 L fermentor. Changes in pressure affect $[CO_2]$ according to equation 4 (Figure 7.4). Since CO_2 solubility is affected, the steady state pH changes (Figure 7.3). Changes in pH due to fluctuations in partial pressure of CO_2 (pCO_2) are sensitive to the alkalinity of the solution (Stumm and Morgan, 1981). Hence, measured pressure and pH changes were used as inputs to the model for predicting the pressure effects. Industrial fermentors are often pressurized up to 1.5–2 atm for dissolved oxygen control during the later stages of the fermentation. Clogging of air filters could also result in pressure changes. When there is a change in head space pressure it is important to apply an appropriate correction to the measured CO_2 values. In airlift fermentors and large stirred tank fermentors, the organisms are exposed to a fluctuating pressure environment, generally in the range 1 – 2 atm. This leads to

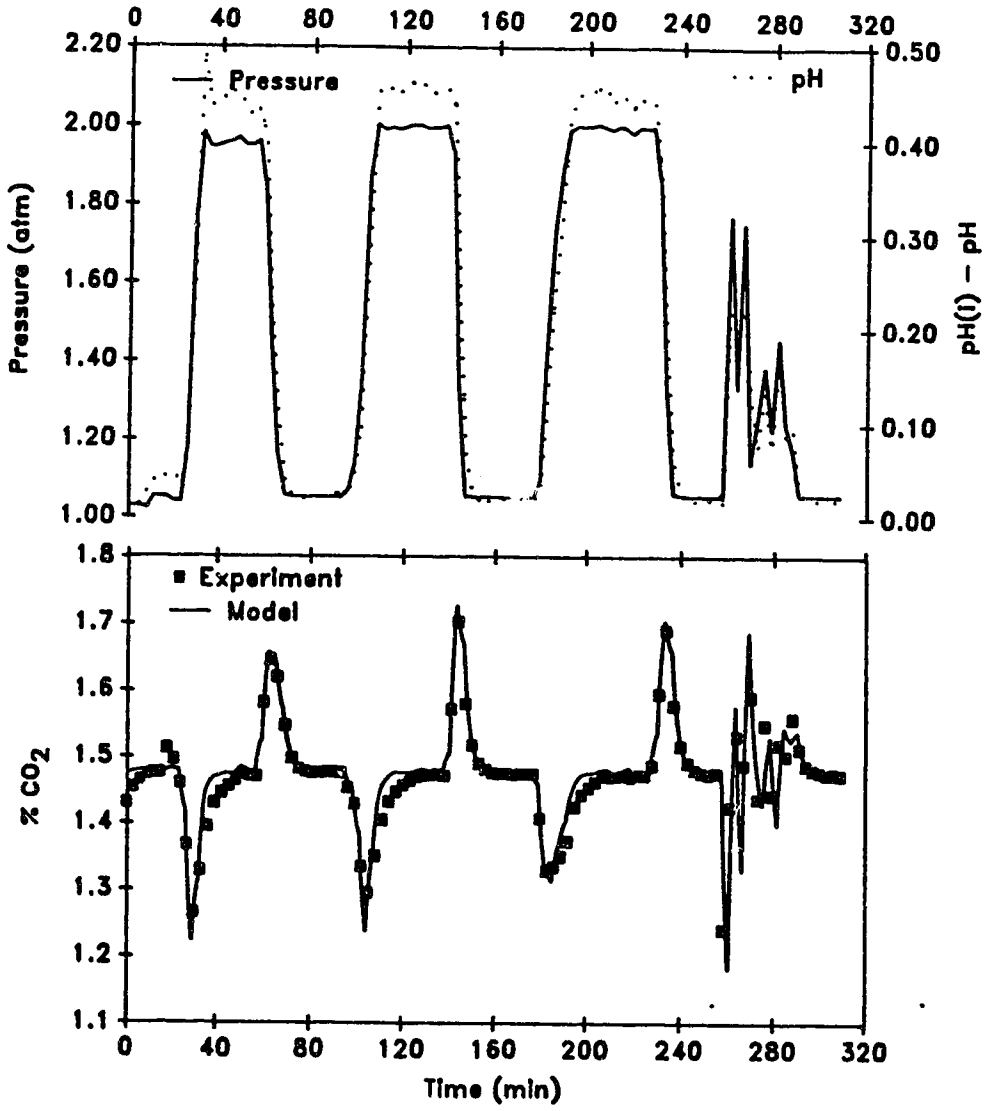

Figure 7.3 Effect of pressure fluctuations on medium pH and $\%CO_2$ in off–gas, experimental data and model prediction.

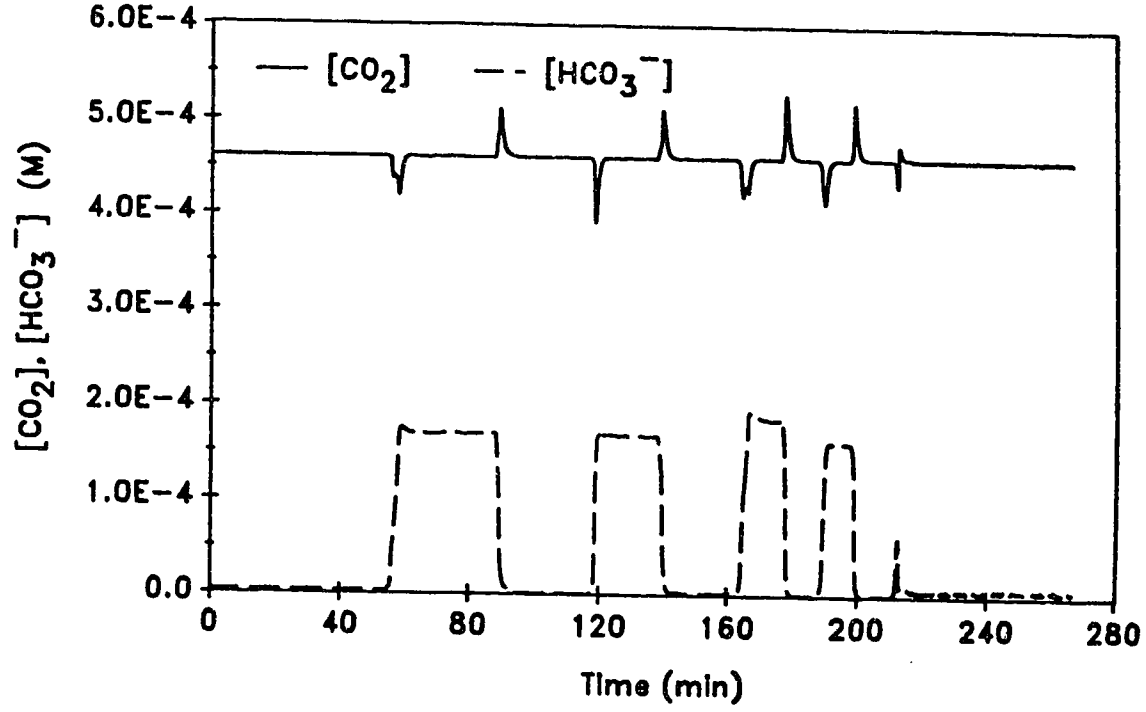

Figure 7.4 Model prediction of $[CO_2]$ and $[HCO_3^-]$ during pressure fluctuations.

increases in [CO_2] (Figure 7.4) and changes in pH in the microenvironment of the cells. Although the resulting pH changes may not be sufficient to affect the cells, the detrimental effects of increases in dissolved CO_2 above 0.1 atm on some microbial species are well known (Ho *et al.*, 1987, Jones and Greenfield, 1982). It is important, therefore to consider effective transfer of CO_2 from the fermentation broth to the gas phase during process scale–up.

Small pressure fluctuations (0.1–0.2 atm) induced larger changes in CO_2 concentrations than small fluctuations in pH (0.1–0.2 units), as illustrated in Figures 7.1 and 7.2. The model gives an estimate for transient changes in CO_2 concentrations and the time scales over which it occurs, due to fluctuations in pH and pressure. Since pH and pressure are routinely monitored in fermentations, CER values can be corrected for pH and pressure changes. The CO_2 concentrations obtained during pH and pressure shifts can be either rejected or the algorithm can be used to determine a time scale for averaging of data in order to eliminate the effects of transients.

Table 7.1 shows the time constants of the model equations (7.3–7.5) calculated from eigenvalues for different values of pH and k_la. The time constant t_1 is the dominant (i.e. reflects slower dynamics) of the two and is a strong function of k_la (inversely proportional), therefore k_la controls the kinetics of CO_2 hydration under these conditions. Higher values of k_la would result in rapid equilibration in response to changes in pH, in agreement with previous work by Nyiri and Lengyel (1968), who suggested that dissolution of CO_2 was enhanced by a reduction of the gas–liquid film resistance. For constant k_la values, an increase in the steady state pH results in an increase in t_1, slowing the kinetics. The smaller time constant t_2 is independent of k_la, but is a function of pH (Table 7.1). Although t_2 increases with

Table 7.1. Time constants of the model evaluated at different k_la and pH with k_{CO_2}= 0.04 s^{-1}, $k_{H_2CO_3}$= 20 s^{-1}, $K_{H_2CO_3}$= 0.0002 M

k_la (h^{-1})	pH	t_1 (min)	t_2 (min)
25	5	2.497	0.016
50	5	1.249	0.016
75	5	0.832	0.016
100	5	0.625	0.016
50	4.5	1.215	0.005
50	5.5	1.358	0.0466

pH, the magnitude of t_2 is very small compared to t_1 and therefore has little effect on the kinetics of the process.

Conclusions

The simple model for CO_2 hydration kinetics is successful in predicting transients in off gas CO_2 caused by fluctuations in fermentor pH and pressure, in water and standard growth media, using only k_la as an adjustable parameter. Mass transfer effects dominate the dynamics of CO_2 equilibration. The algorithm presented here, along with predetermined k_la values, can be used for correction of CER and RQ, which may then be used for on–line estimation and control of fermentors.

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CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

Effect of DO control on antibiotic production in *S. clavuligerus*

(1) Previous studies have shown the importance of dissolved oxygen levels on antibiotic biosynthesis in *S. clavuligerus* (Rollins *et al.*, 1988, 1990). Control of oxygen enrichment using a proportional integral controller was adequate to maintain dissolved oxygen at 100 % and 50 % , at constant shear and mass transfer conditions. These dissolved oxygen levels were found to have a significant effect on cephamycin C production in *S. clavuligerus*. In fermentations using complex media, a 2.4 fold increase in final cephamycin C yield was obtained when dissolved oxygen was maintained at 100 % saturation during the growth phase.

(2) In a previous study using defined media, maintaining dissolved oxygen at saturation levels was detrimental to antibiotic production resulting in lower yields of cephamycin C (Rollins *et al.*, 1989). Hence, the composition of the fermentation medium seems to regulate the effect of oxygen on antibiotic biosynthesis. From the present state of knowledge it is not clear whether the regulatory effects of oxygen operate at the genetic level by affecting the promoters responsible for the biosynthetic enzymes, or are related to the direct or indirect interaction with the biosynthetic enzymes. Further work is necessary to determine the mechanism by which oxygen affects the enzyme activities in the presence of different carbon and nitrogen sources. An understanding of this mechanism could lead to further developments in rational strategies for determining dissolved oxygen and nutrient levels in the fermentation environment which would enhance antibiotic production. Meanwhile, experimental data on the effects of dissolved oxygen levels on biosynthetic enzyme activities and antibiotic yields in different media would

help in the development of mathematical models which can be used for profiled control of dissolved oxygen levels to maximize production.

(3) In the absence of information on shear effects on cell damage and antibiotic production, using gas mixtures to control dissolved oxygen levels even at low values seems to be a feasible approach, at least for experimental studies.

Experimental simulation of DO fluctuations using a small fermentor

(1) In large production fermentors, as a result of imperfect mixing the microbes are exposed to continuous fluctuations in dissolved oxygen and other physical parameters. In case of antibiotic production, these fluctuations in dissolved oxygen levels could play an important role in determining product yields. Previous experimental methods using a two fermentor system or a fermentor with a recycle loop are not adequately flexible to simulate a given circulation time distribution on a small scale. The Monte Carlo method, presented in this study is a rational experimental technique for duplicating the large scale circulation time distribution on a small scale. Unlike previous methods used to simulate fluctuations in large fermentor using a small fermentor, this method is flexible enough to simulate any given circulation time distribution in a small fermentor.

(2) Experiments to study the effects of dissolved oxgyen fluctuations on primary metabolism in *Saccharomyces cerevisiae* and secondary metabolism in *Streptomyces clavuligerus* gave reproducible results. In fed–batch fermentations of *S. cerevisiae* lower biomass production and higher ethanol accumulation was observed. Lower cephamycin C synthesis was observed in batch fermentations of *S. clavuligerus*. These results indicate that the distribution of circulation times, along with the mean circulation time is an important parameter to be considered in fermentation scale–up. The use of

improved bulk circulation impellers such as the Prochem results in a circulation time distribution with a lower standard deviation (Buckland *et al.*, 1988). This change in distribution could be the reason for the improved performance of such impellers for viscous fermentation processes.

(3) In this study, aeration was assumed to be in the vicinity of a single impeller, resulting in a single micromixed zone, which is a conservative assumption. The method could be extended to handle multiple impellers and multiple micromixed zones. The most effective way of obtaining the circulation time distribution seems to be by direct measurement. Data on circulation time distribution in large fermentors is a limitation to the use of this method. In order to determine the accuracy with which the method is able to predict large scale yields, the actual circulation time distribution from a large fermentor must be used to carry out the small scale experiments. The results from these experiments must then be compared with actual large scale fermentations. In large fermentors the cells would experience high dissolved oxygen levels and shear in the impeller region. A further step would be to simulate simultaneously the fluctuations experienced by the cells in dissolved oxygen and shear. This would require the quantification of shear in stirred tanks with turbulent flow. Overall, the Monte Carlo approach of using a small fermentor to simulate the fluctuations in physical parameters experienced in large fermentors is promising for assessing the robustness of a cell line for use on the production scale.

(4) Autocorrelation functions can be used to characterize the Monte Carlo and periodic aeration signals. The autocorrelation functions can be analysed to determine their possible use in placing bounds on performance during scale–up.

Effect of pH and pressure on CO_2 monitoring of fermentation off–gases

(1) In addition to oxygen uptake rate, the carbon dioxide evolution rate is an important on–line measurement for state estimation and process control. In this study the CO_2 level was used to determine the time for switching of dissolved oxygen setpoints in the dissolved oxygen control experiments. Changes in the pH and pressure levels in the fermentor, however, affect the dynamic CO_2 equilibrium in aqueous fermentation broths. A model using k_1a as the adjustable parameter was successful in predicting transient responses in fermentation off–gas CO_2 caused by changes in fermentor pH and pressure. Mass transfer characteristics influence the time scale of these transient responses. A simple averaging scheme would be adequate to filter out the transients before using CER or RQ for on–line estimation and control.

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APPENDIX A

MATHEMATICAL EXPRESSIONS FOR CALIBRATION CURVES

Table A.1 **Dry cell weight vs optical density calibration curves**

Saccharomyces cerevisiae (Chapter 5)

$$DCW\ (g/L) = 0.98046\ (OD_{620} - 0.028) \qquad r^2 = 0.981$$

Streptomyces clavuligerus (Defined media, Chapter 3)

$$DCW\ (g/L) = 0.515\ (\ OD_{660}) - 0.078 \qquad r^2 = 0.966$$

Streptomyces clavuligerus (Complex media, Chapter 4,6)

Exponential growth phase

$$DCW\ (g/L) = 0.848\ (\ OD_{660}) - 0.515 \qquad r^2 = 0.985$$

Lysis phase

$$DCW\ (g/L) = 1.096\ (\ OD_{660}) - 2.837 \qquad r^2 = 0.934$$

Table A.2 Calibration curves for chemical assays

Glycerol determination by GC analyses (Chapter 3)

$$\text{Weight ratio (glycerol/hexanol)} = 5.44\ (\text{Area ratio}) + 0.868 \qquad r^2 = 0.912$$

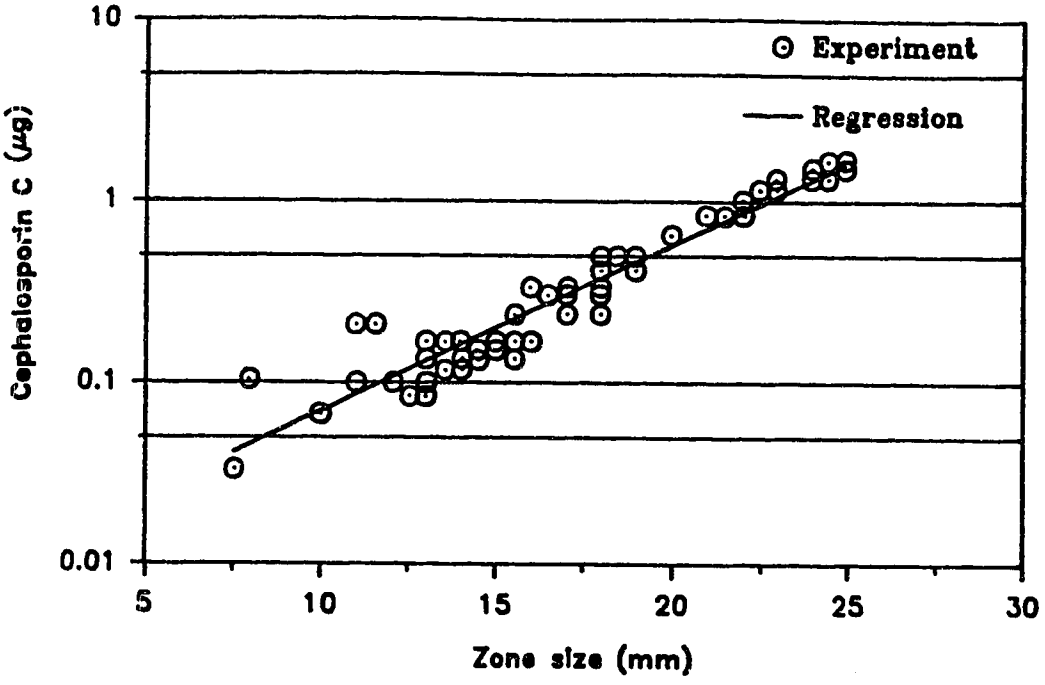
Asparagine determination by ninhydrin assay (Chapter 3)

$$\text{Asparagine (mg)} = 1.11\ (OD_{570}) + 0.318 \qquad r^2 = 0.958$$

Protein assay by Bio–Rad method using Bovine Serum Albumin (BSA) as standard (Chapter 3)

$$\text{Proteins } (\mu g) = 117.02\ (OD_{595}) - 48.87 \qquad r^2 = 0.987$$

Figure A.1 Antibiotic bioassay standard curve for cephalosporin C.



LOG [Cephalosporin C (μg)] = 0.0913 (zone mm) – 2.069

$r^2 = 0.956$

APPENDIX B

DATA FROM REPEATED EXPERIMENTS : *Saccharomyces cerevisiae*

Table B.1 Description of *S. cerevisiae* experiments

Run	Experiment
Scerv_9	
Scerv_10	Batch experiment with continuous aeration
Scerv_11	
Scerv_12	Batch experiment with Monte Carlo on/off aeration
Scerv_13	
Scerv_14	Batch experiment with Monte Carlo air/N_2 aeration
Scerv_20	
Scerv_21	Constant fed–batch experiment with continuous aeration
Scerv_22	
Scerv_23	Constant fed–batch experiment with Monte Carlo aeration
Scerv_24	
Scerv_25	Constant fed–batch experiment with periodic aeration

Table B.2 Experiment Scerv_9 : Biomass and Ethanol

T(h)	DCW(g/L)	Ethanol(g/L)
0	0.047	-
2	0.1	3.44
4	0.23	2.97
6	0.58	2.99
8	1.21	2.9
10	2.24	2.3
12	2.72	2.57
14	3.24	2.1
16	3.67	1.54
18	4.28	0.7
20	4.53	0.33
22	4.8	0.1

Table B.3 Experiment Scerv_10 : Biomass and Ethanol

T(h)	DCW(g/L)	Ethanol(g/L)
0	0.041	-
2	0.18	3.35
4	0.27	3.1
6	0.61	2.89
8	1.25	2.89
10	2.3	2.4
12	2.8	2.51
14	3.31	2.2
16	3.78	1.55
18	4.31	0.8
20	4.6	0.35
22	4.86	0.15

Table B.4 Experiment Scerv_11 : Biomass and Ethanol

T(h)	DCW(g/L)	Ethanol(g/L)
0	0.042	-
3	0.13	3.17
6	0.51	2.87
8	1.02	2.62
10	1.93	2.74
13	2.31	2.74
16	2.96	2.32
19	3.75	1.39
22	4.6	0.67
24	4.61	0.1

Table B.5 Experiment Scerv_12 : Biomass and Ethanol

T(h)	DCW(g/L)	Ethanol(g/L)
0	0.042	-
3	0.11	3
6	0.53	2.76
8	1	2.58
10	1.95	2.7
13	2.37	2.65
16	2.9	2.3
19	3.78	1.34
22	4.5	0.61
24	4.55	0.14

Table B.6 Experiment Scerv_13 : Biomass and Ethanol

T(h)	DCW(g/L)	Ethanol(g/L)
0	0.042	-
2	0.1	3.41
4	0.2	0.64
6	0.56	2.58
8.25	1.28	2.69
10	2.14	2.47
12	2.52	2.74
14	2.82	2.28
17	3.13	1.98
19	3.76	1.21
21	4.26	0.82
24	4.33	0.52

Table B.7 Experiment Scerv_14 : Biomass and Ethanol

T(h)	DCW(g/L)	Etnanol(g/L)
0	0.042	-
2	0.15	3.39
4	0.23	0.61
6	0.53	2.4
8	1.35	2.7
10	2.1	2.45
12	2.46	2.69
14	2.76	2.25
17	3.1	1.88
19	3.68	1.18
21	4.2	0.79
24	4.38	0.46

Table B.8 Experiment Scerv_20
Biomass, Ethanol and Glucose

T(h)	DCW (g/L)	Ethanol (g/L)	Glucose (g/L)
0	2.24	2.39	0.37
3	3.68	4.01	0.095
6	5.87	3.08	0.12
9	9.15	2.91	0.16
12	12.4	0.88	0.145
15	14.92	0.28	0.3

Table B.9 Experiment Scerv_21
Biomass, Ethanol and Glucose

T(h)	DCW (g/L)	Ethanol (g/L)	Glucose (g/L)
0	2.21	2.48	0.34
3	3.59	3.9	0.14
6	5.76	3.1	0.16
9	9.2	2.84	0.19
12	12.15	0.94	0.15
15	14.56	0.23	0.35

Table B.10 Experiment Scerv_22
Biomass, Ethanol and Glucose

T(h)	DCW (g/L)	Ethanol (g/L)	Glucose (g/l)
0	2.23	2.84	0.08
3	2.95	2.72	0.11
7	6.5	2.9	0.12
9	7.77	4.32	0.4
12	10.32	3.14	0.17
15	11.97	2.5	0.29

Table B.11 Experiment Scerv_23
Biomass, Ethanol and Glucose

T(h)	DCW (g/L)	Ethanol (g/L)	Glucose (g/L)
0	2.2	2.9	0.09
3	3.1	2.65	0.16
7	6.37	2.76	0.15
9	7.98	4.4	0.45
12	10.4	3.2	0.23
15	12.33	2.5	0.34

Table B.12 Experiment Scerv_24
Biomass, Ethanol and Glucose

T(h)	DCW (g/L)	Ethanol (g/L)	Glucose (g/L)
0	2.26	3.46	0.08
3	3.77	2.51	0.085
7	5.73	2.43	0.07
9	7.77	3.37	0.13
12	9.42	2.4	0.14
15	10.36	2.42	0.26

Table B.13 Experiment Scerv_25
Biomass, Ethanol and Glucose

T(h)	DCW (g/L)	Ethanol (g/L)	Glucose (g/L)
0	2.1	3.1	0.1
3	2.9	2.56	0.19
7	6.23	2.65	0.12
9	7.65	4.2	0.4
12	10.4	3.1	0.26
15	10.6	2.53	0.3

Figure B.1 Experiment Scerv_9

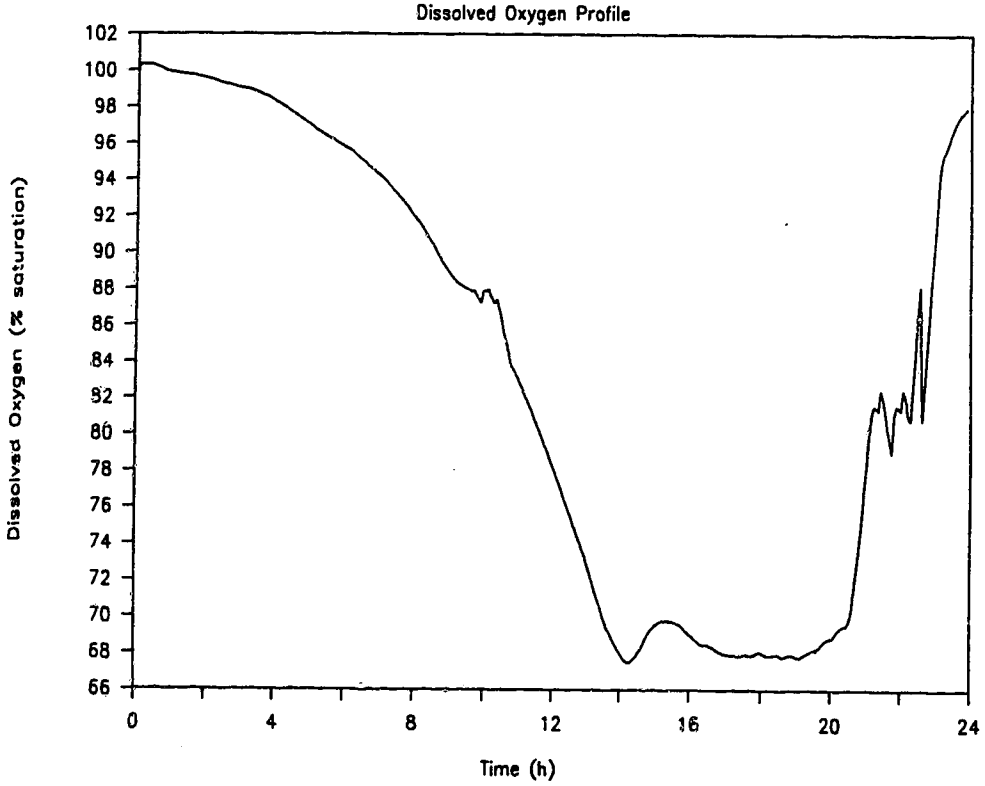


Figure B.2 Experiment Scerv_10

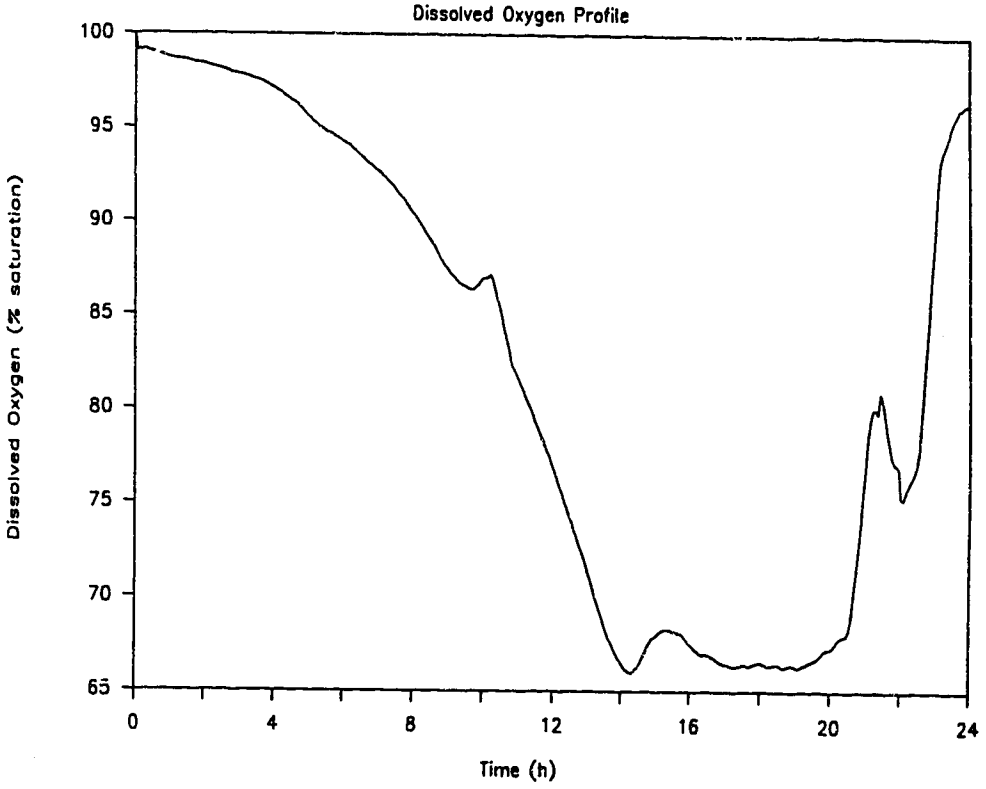


Figure B.3 Experiment Scerv_11

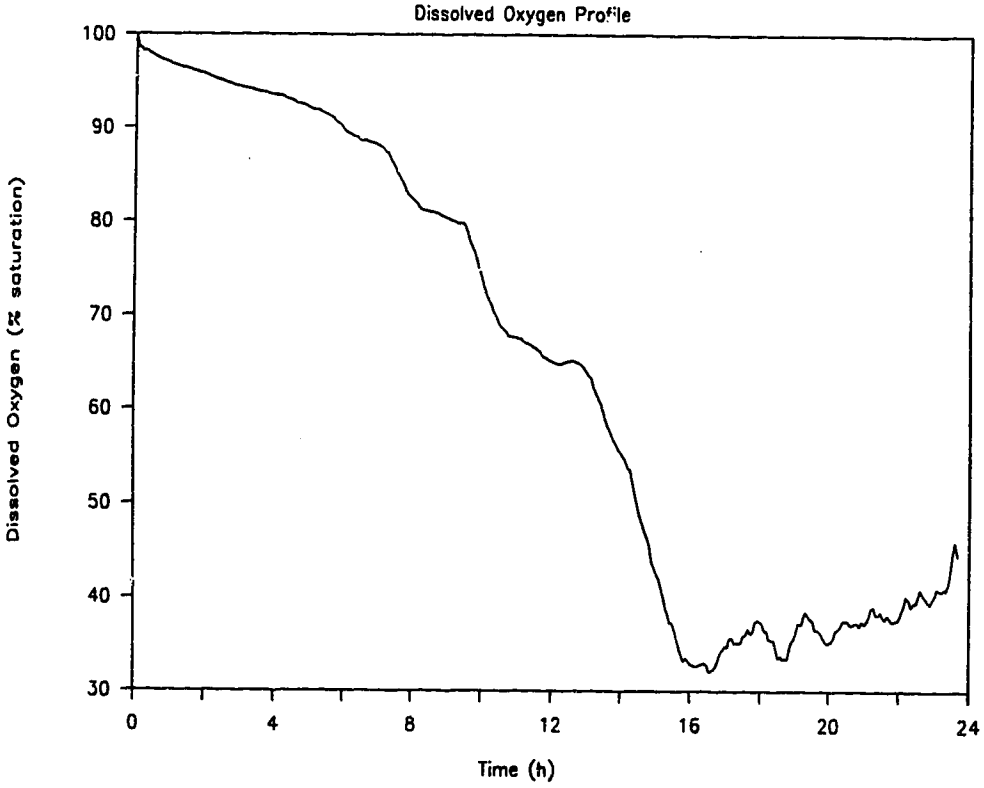


Figure B.4 Experiment Scerv_12

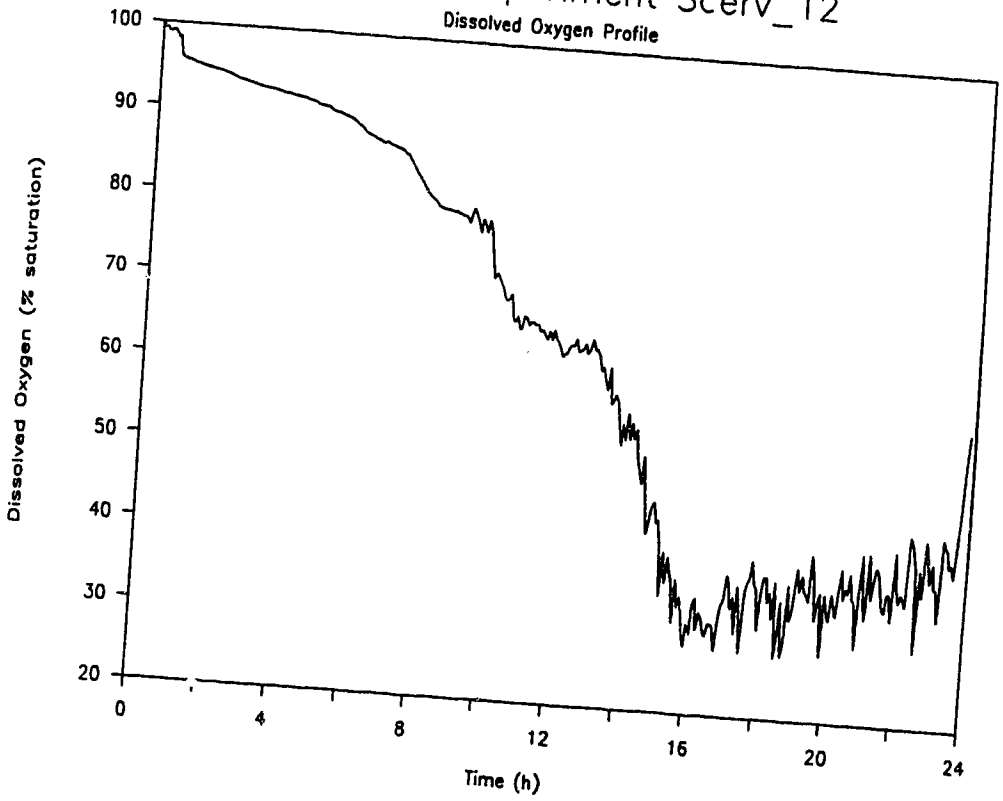


Figure B.5 Experiment Scerv_13

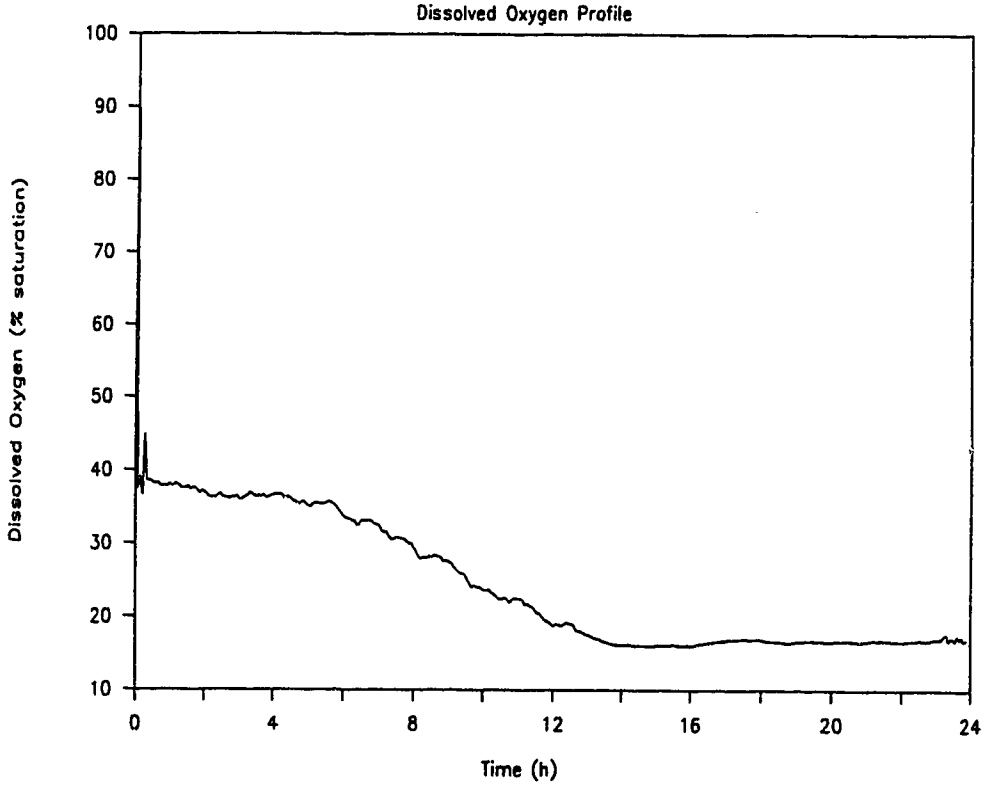


Figure B.6 Experiment Scerv_14

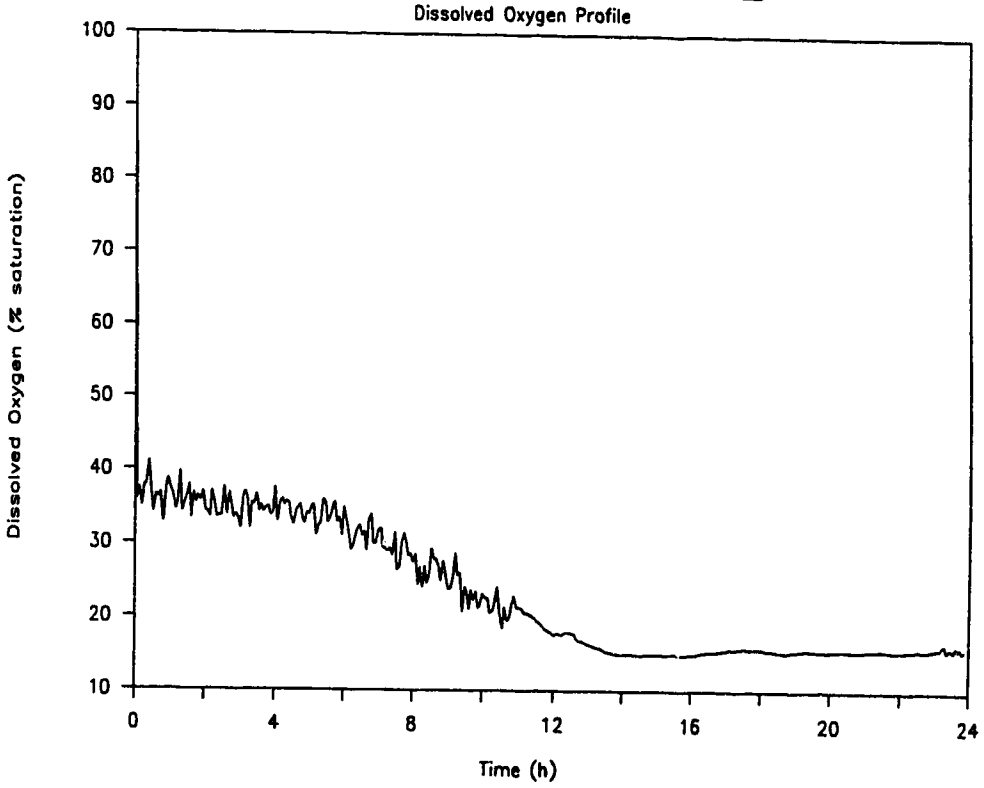


Figure B.7 Experiment Scerv_20

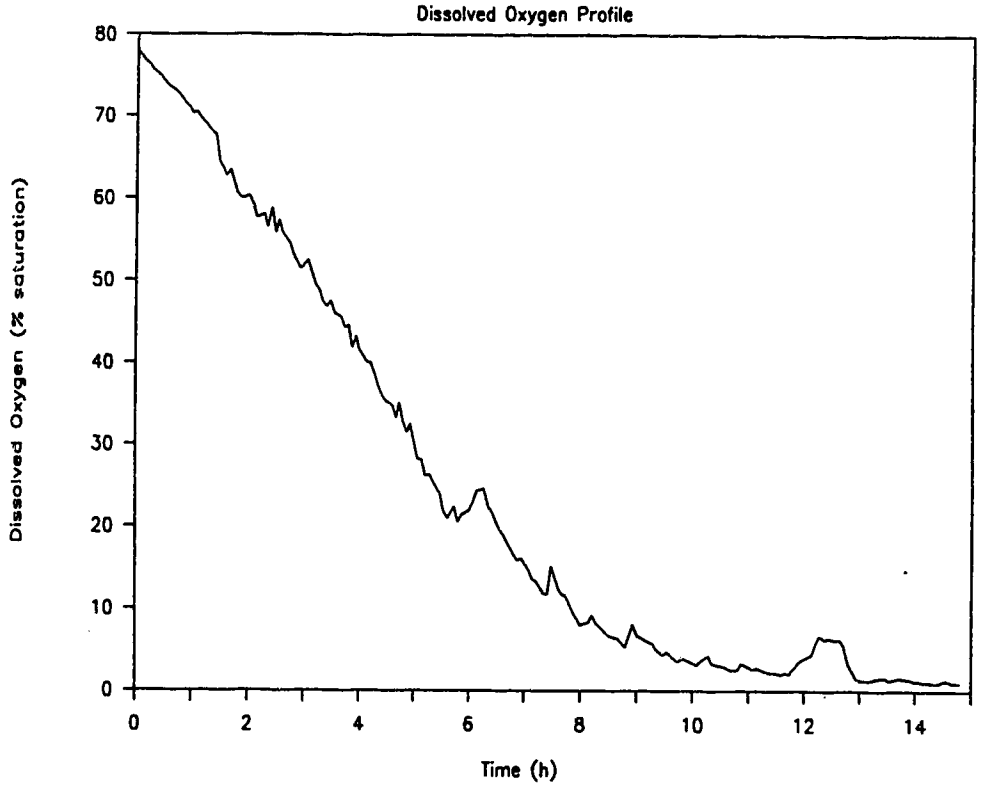


Figure B.8 Experiment Scerv_21

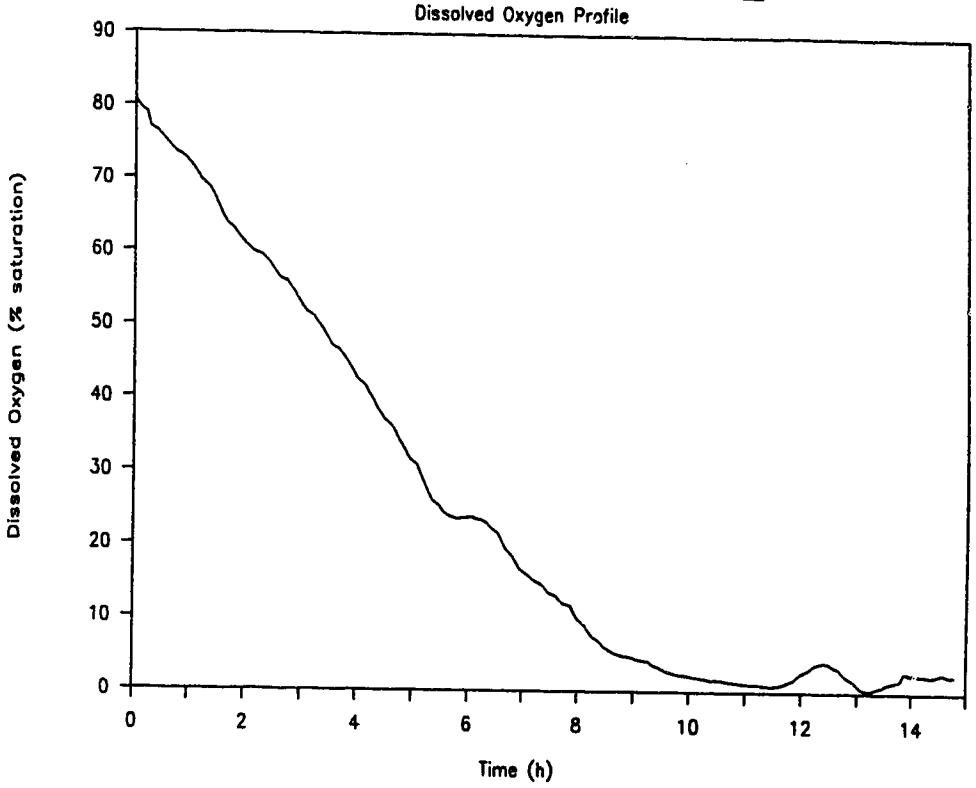


Figure B.9 Experiment Scerv_22

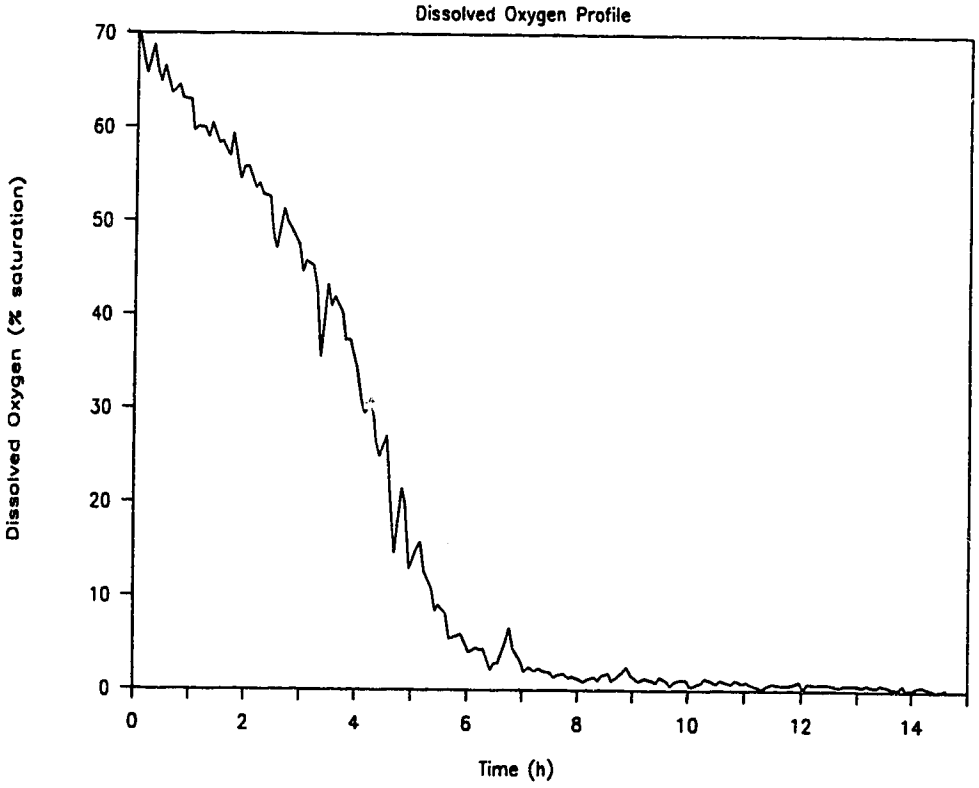


Figure B.10 Experiment Scerv_23

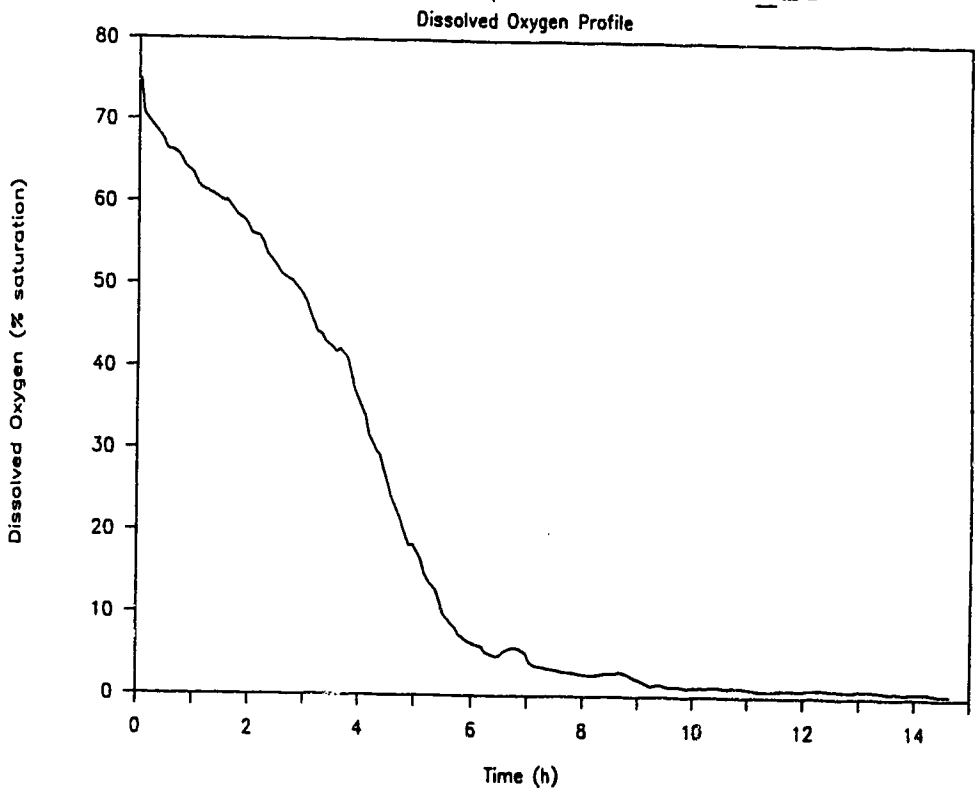


Figure B.11 Experiment Scerv_24

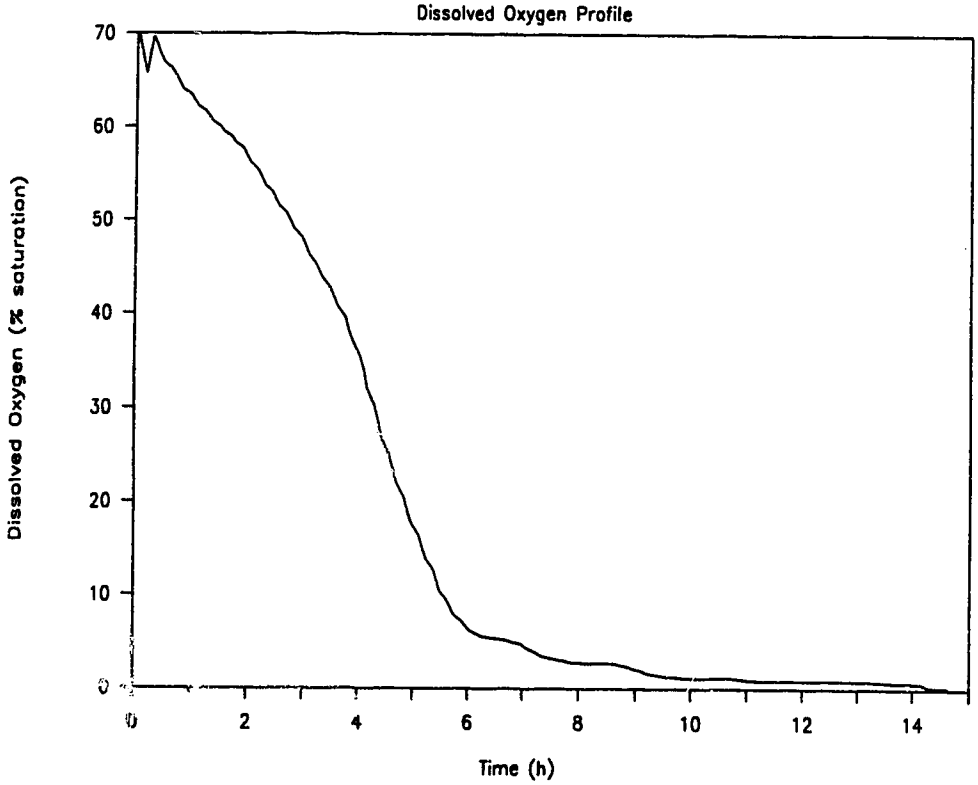
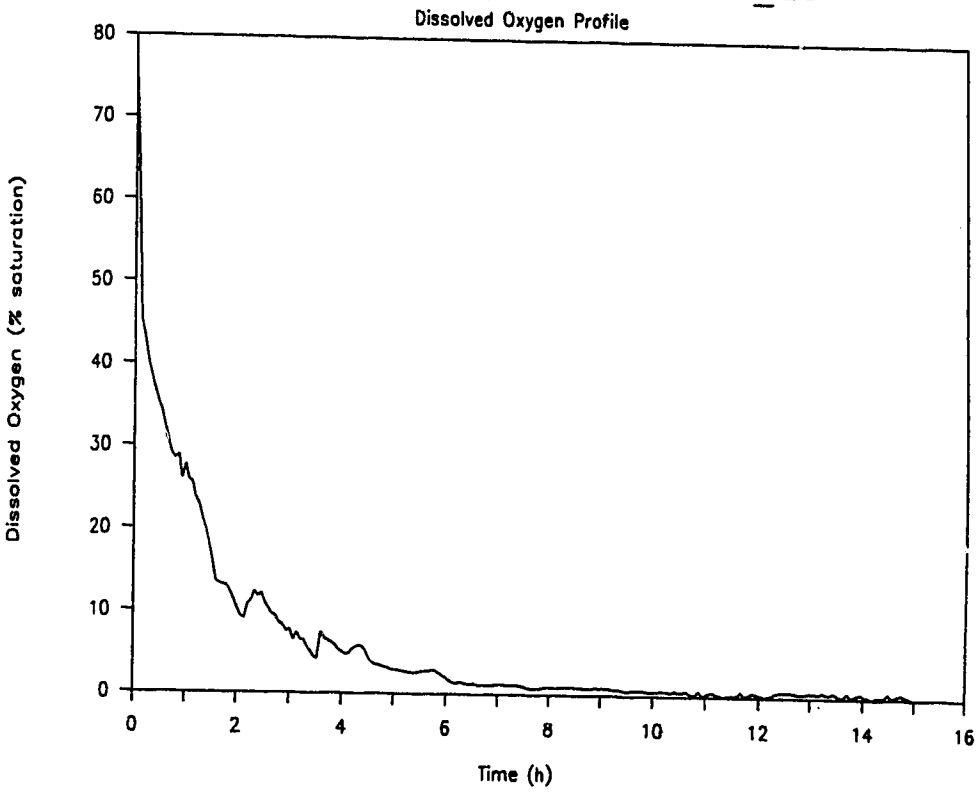


Figure B.12 Experiment Scerv_25



APPENDIX C

DATA FROM REPEATED EXPERIMENTS : *Streptomyces clavuligerus*

Table C.1 Description of *S. clavuligerus* experiments

Run	Experiment
Sclav_7, 8, 9	Batch experiment with continuous aeration
Sclav_10, 11, 12	Batch experiment with Monte Carlo controlled aeration
Sclav_13, 14, 15	Batch experiment with periodic aeration
Sclav_16	Batch experiment without DO control
Sclav_17	Batch experiment with DO control at 100 % throughout the experiment
Sclav_18	Batch experiment with DO control at 50 % throughout the experiment
Sclav_19	Batch experiment with DO control at 100 % during growth phase
Sclav_20	Batch experiment with DO control at 100 % during growth, 50 % later

Table C.2 Experiment Sclav_7
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
10	0.3	0.01	0	0	0
14	1.01	0.29	0	0	0
16	2.1	0.69	0	0	0
18.5	3.65	0.54	0	0	0
22	6.1	2.36	0	0	0
27	6.87	2.22	0	0	0
33.5	5.71	1.75	1.75	1.3	0.45
38.5	4.52	0.84	3.2	1.75	1.45
42	3.93	1.02	4	2.25	1.75
47	3.41	0.84	6	4	2
58	3.12	0.67	15	7	8
62.5	2.45	0.57	30	13.75	16.25
65.5	2.57	0.69	45	22.25	22.75
72	2.3	0.57	67.5	32.5	35
84	1.69	0.46	82.5	50	32.5
90	1.21	0.51	85	70	15
97	1.12	-	87	72	15
98	1.1	-	91.4	73	18.4

Table C.3 Experiment Sclav_8
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins (g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
9.5	0.35	0.01	0	0	0
14	1.2	0.35	0	0	0
16	1.26	0.73	0	0	0
18.5	3.96	0.6	0	0	0
22	6.23	2.59	0	0	0
27	7.13	2.36	1.5	0	1.5
33	5.8	1.9	2	1.2	0.8
38	4.7	0.93	3.1	1.8	1.3
42	4.1	1.1	5	2.4	2.6
47	3.45	0.91	7	5	2
58	3.2	0.76	17	8	9
62.5	2.5	0.61	32	14.5	17.5
65.5	2.7	0.72	46	25	21
72	2.46	0.62	71	33	38
84	1.84	0.53	85	45	40
90	1.3	0.54	85	50	35
97	1.2 -		89	56	33
98	1.25 -		96	56	40

Table C.4 Experiment Sclav_9
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins (g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
8	0.3	0.01	0	0	0
13	1	0.35	0	0	0
16	1.3	0.73	0	0	0
19	4.1	0.6	0	0	0
22	6.4	2.59	0	0	0
27	7.6	2.36	1.5	0	1.5
33	6.2	1.9	2	1.2	0.8
38	5.1	0.93	3.5	1.8	1.7
42	4.6	1.1	7	3	4
47	3.7	0.91	10	7	3
58	3.4	0.76	20	10	10
62.5	2.6	0.61	35	15	20
65.5	2.8	0.72	50	30	20
72	2.5	0.62	55	35	20
84	2.1	0.53	70	40	30
90	1.4	0.54	75	50	25
97	1.3	-	80	55	25
98	1.15	-	85	55	30

Table C.5 Experiment Sclav_10
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins (g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
6	0.36	0.03	0	0	0
18.5	4.65	0.87	0	0	0
21	5.9	1.5	0	0	0
24	6.74	-	0	0	0
27	8.14	2.14	0	0	0
30	7.5	2	0	0	0
33	7.7	2.22	2.35	0	2.35
41.5	2.75	0.67	3	0	3
47	1.8	0.59	3.8	0	3.8
50.5	2	0.52	5.6	0	5.6
56.5	1.88	0.43	8.75	3.5	5.25
65.5	2.2	0.37	11.25	4.5	6.75
70.5	1.96	0.38	13.75	5.5	8.25
74	1.7	0.33	23.75	9.5	14.25
80.5	1.52	0.24	46.25	18.5	27.75
92	0.86	0.25	52.5	21	31.5
96	0.97	0.63	75	30	45
98	0.98	0.6	78	31	47

Table C.6 Experiment Sclav_11
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins (g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
6	0.4	0.02	0	0	0
18	4.1	0.9	0	0	0
21	5.4	1.5	0	0	0
24	6.5	2.1	0	0	0
27	7.4	2.2	0	0	0
30	7.5	2.3	0	0	0
33	7.6	2.4	4	0	4
42	4.6	0.9	5	0	5
47	3.2	0.8	5	0	5
51	2.6	0.65	8	2.5	5.5
57	2.3	0.45	14	4	10
65	2.2	0.5	23	7	16
71	2.1	0.41	36	10	26
74	1.5	0.4	54	13	41
81	1.4	0.2	60	30	30
92	0.9	0.2	70	25	45
96	1	0.57	75	31	44
98	1.2	0.6	75	34	41

Table C.7 Experiment Solav_12
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins (g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
7	0.5	0.03	0	0	0
15	3.5	1.1	0	0	0
21	5.6	1.4	0	0	0
25	6.4	1.9	0	0	0
27	7.5	2.3	0	0	0
30	7.7	2.4	0	0	0
33	7.9	2.5	4	0	4
42	5.8	2.2	5	0	5
47	4.5	0.9	6	1.2	4.8
51	3.6	0.7	12	4.5	7.5
57	2.5	0.5	16	5	11
65	2.4	0.55	30	8	22
71	2.1	0.45	45	12	33
74	1.6	0.43	55	16	39
81	1.4	0.3	65	30	35
92	1.2	0.35	75	30	45
96	1.1	0.6	79	35	44
98	0.9	0.65	80	40	40

Table C.8 Experiment Sclav_13
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins (g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
8.5	0.18	0.1	0	0	0
12	1.16	0.65	0	0	0
15.5	1.77	1.5	0	0	0
21	5.34	2.95	0	0	0
25	5.72	3.02	0	0	0
27	6.26	2.9	0	0	0
33.75	6.05	1.46	0	0	0
38	5.25	1.16	0	0	0
42	3.77	0.96	0	0	0
47.5	3.47	0.74	6.6	3.45	3.15
57	3.37	0.61	8.4	3.75	4.65
63	3.24	0.63	10.3	4.75	5.55
66.5	3.24	0.59	12	6	6
72	2.7	0.55	26	14	12
82	2.18	0.53	65	31.25	33.75
87.5	2.02	0.57	74	36.25	37.75
92	2.34	0.46	73	36	37
97	1.79	0.5	73	36	37

Table C.9 Experiment Sclav_14
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins (g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
8	0.24	0.1	0	0	0
12	1.4	0.6	0	0	0
16	1.85	1.5	0	0	0
21	5.5	2.9	0	0	0
25	5.8	3.1	0	0	0
27	6.5	3.3	0	0	0
34	7.1	3.4	0	0	0
38	6.5	1.3	0	0	0
42	4.9	0.9	0	0	0
49	3.8	0.8	7	3.5	3.5
57	3.5	0.7	8.6	4	4.6
63	3.3	0.65	10.3	5.6	4.7
67	3.2	0.6	25	18	7
72	2.8	0.65	45	27	18
82	2.2	0.56	70	32	38
87.5	2.1	0.61	74	37	37
92	2.1	0.51	75	40	35
97	1.8	0.6	75	41	34

Table C.10 Experiment Sclav_15
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins (g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
9	0.3	0.12	0	0	0
13	1.5	0.54	0	0	0
16	1.9	1.6	0	0	0
21	5.6	2.7	0	0	0
25	6	3.2	0	0	0
27	6.9	3.4	0	0	0
34	7.4	3.6	0	0	0
39	6.6	2	0	0	0
42	5.2	1.2	0	0	0
49	4.1	1	7	3	4
57	3.7	0.8	9	4	5
63	3.5	0.7	12	7	5
67	3.1	0.8	30	20	10
72	2.9	0.71	50	29	21
82	2.1	0.6	69	35	34
87.5	2.1	0.7	75	39	36
92	2.1	0.73	78	45	33
97	1.9	0.75	79	49	30

Table C.11 Experiment Sclav_16
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins (g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
8	0.41	0.14	0	0	0
11.5	1.58	0.53	0	0	0
14.5	4.4	1.37	0	0	0
17.5	7.48	2.58	0	0	0
[illegible]	8.65	1.95	1.7	1.3	0.4
[illegible]	7.68	1.49	3.2	1.75	1.45
36	6.06	1.39	4	2.25	1.75
40	5.2	1.21	6	4	2
[illegible]	5.12	0.97	15	7	8
47	4.85	1.15	30	13.75	16.25
[illegible]	4.66	0.79	45	22.25	22.75
[illegible]	4.1	0.77	67.5	32.5	35
64.[illegible]	3.56	0.67	82.5	50	32.5
70.5	3.5	0.55	85	70	15
83.5	2.78	-	89	73	16
89	2.32	0.49	89	73	16
96	2.38	0.39	89	73	16

Table C.12 Experiment Sclav_17
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins (g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
8	0.41	0.32	0	0	0
11.5	1.58	0.8	0	0	0
14.5	4.4	0.72	0	0	0
17.5	7.48	1.52	0	0	0
22.5	8.65	1.6	1.7	1.29	0.41
31.5	7.68	1.09	3.2	1.75	1.45
36	6.07	1.38	4	2.25	1.75
40	5.18	1.3	6	4	2
42.5	5.11	1.13	15	7	8
47	4.85	0.95	30	13.75	16.25
55.5	4.66	0.93	45	22.25	22.75
59.5	4.1	0.93	67.5	32.5	35
64.5	3.56	0.9	82.5	50	32.5
70.5	3.5	0.79	85	70	15
83.5	2.78	-	89	73	16
89	2.32	-	89	73	16
96	2.38	-	89	73	16

Table C.13 Experiment Sclav_18
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins (g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
9.5	0.93	0.28	0	0	0
13	3.03	0.96	0	0	0
15	2.67	2.48	0	0	0
17	5.1	2.5	0	0	0
20	6.1	2.11	0	0	0
24.5	7.5	2.27	1.7	0.68	1.02
28	8.4	1.97	1.7	0.77	0.93
35	8.37	1.29	1.7	1.1	0.6
39	4.72	1.26	4.5	1.8	2.7
43	4.72	1.08	7.5	4.1	3.4
48	3.97	0.91	12	6.6	5.4
59	3.74	0.66	15	9.75	5.25
63	3.3	0.59	19.5	10.53	8.97
68.5	2.87	0.62	27.5	19.25	8.25
72.5	2.57	0.8	37.5	30	7.5
86	2.34	0.63	37.5	28.13	9.37
90	2.18	0.49	50	39	11
97.5	1.97	0.47	50	40	10

Table C.14 Experiment Sclav_19
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins (g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
8	0.17	0.11	0	0	0
11	1.29	0.53	0	0	0
13	2.38	0.9	0	0	0
15	3.86	1.51	0	0	0
17.5	6.56	1.99	0	0	0
20.5	8.24	2.34	0	0	0
22.5	8.88	2.1	12	7.2	4.8
31.5	8.34	1.9	45	31.5	13.5
35.5	7.35	1.65	65	48.75	16.25
39	6.23	1.14	85	68	17
42.5	5.64	1.15	100	85	15
47	5.08	1	125	93.75	31.25
58	3.99	0.77	150	127.5	22.5
63.5	4.52	0.9	180	135	45
70.5	3.81	0.647	190	152	38
80.5	3.44	0.57	195	171.6	23.4
85	3.72	0.64	195	163.8	31.2
89.5	3.24	0.44	195	165.75	29.25
95	2.91	0.42	195	165.75	29.25

Table C.15 Experiment Sclav_20
Biomass, Proteins and Antibiotics

T(h)	DCW (g/L)	Proteins (g/L)	Total Abtcs (mg/L)	Ceph C (mg/L)	Pen N (mg/L)
0	0.001	0	0	0	0
8	0.17	0.15	0	0	0
11	1.3	0.6	0	0	0
13	2.31	0.9	0	0	0
15	3.8	1.3	0	0	0
17.5	6.4	2.1	0	0	0
21	8.2	2.3	0	0	0
22.5	8.9	2.8	10	6.9	3.1
31.5	8.2	1.8	40	29	11
36	7.4	1.7	62	47.5	14.5
39	6.1	1.12	81	66.7	14.3
43	5.55	1.24	95	81	14
47	5.01	1.1	119	92	27
56	4.1	0.9	144	124	20
63.5	4.4	1.1	170	132	38
70.5	3.72	0.72	181	147	34
80.5	3.356	0.61	190	169.7	20.3
85	3.65	0.71	190	169.7	20.3
89.5	3.12	0.5	190	169.7	20.3
95	2.8	0.46	190	169.7	20.3

Figure C.1 Experiment Sclav_7

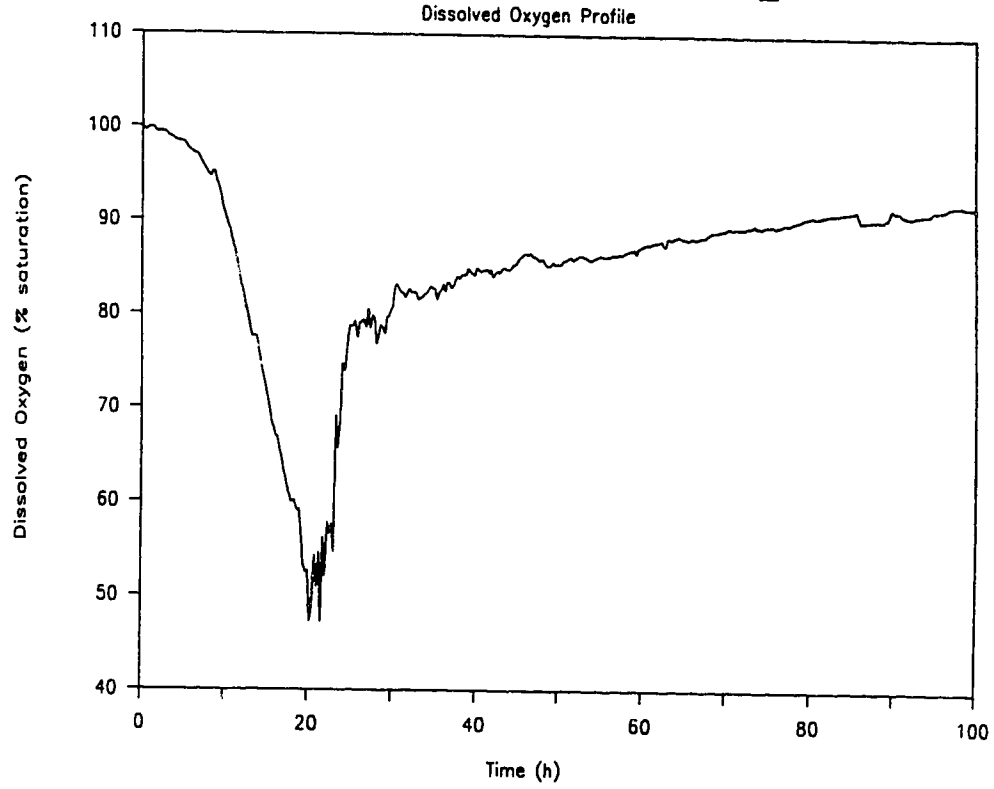


Figure C.2 Experiment Sclav_8

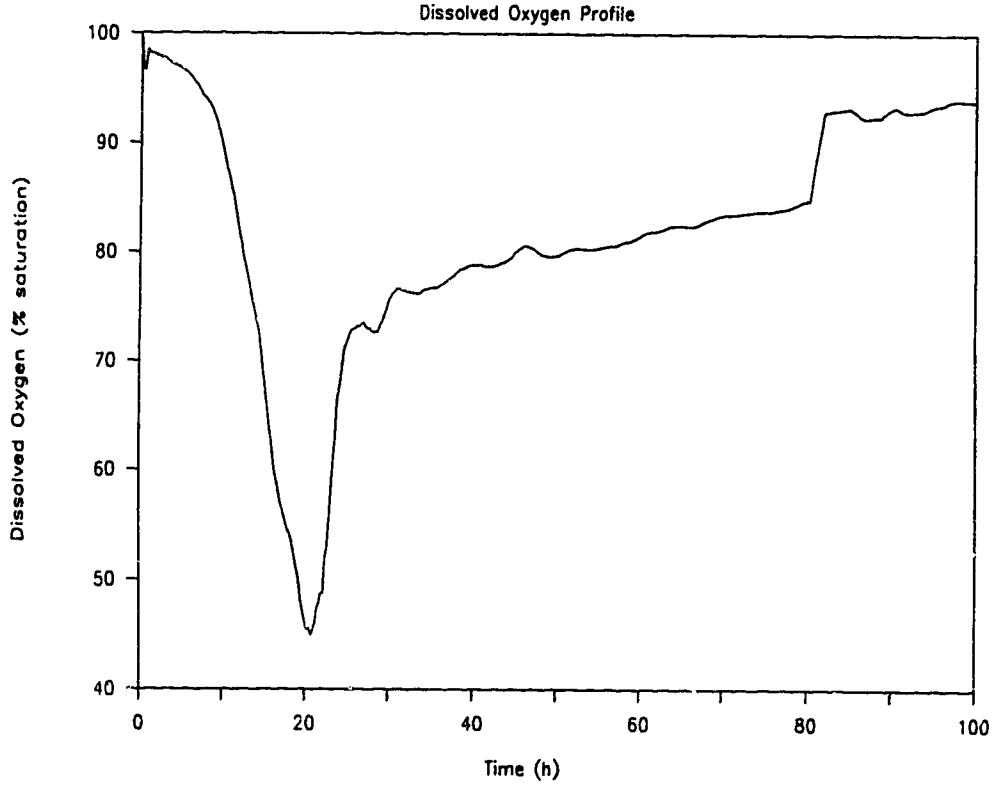


Figure C.3 Experiment Sclav_9

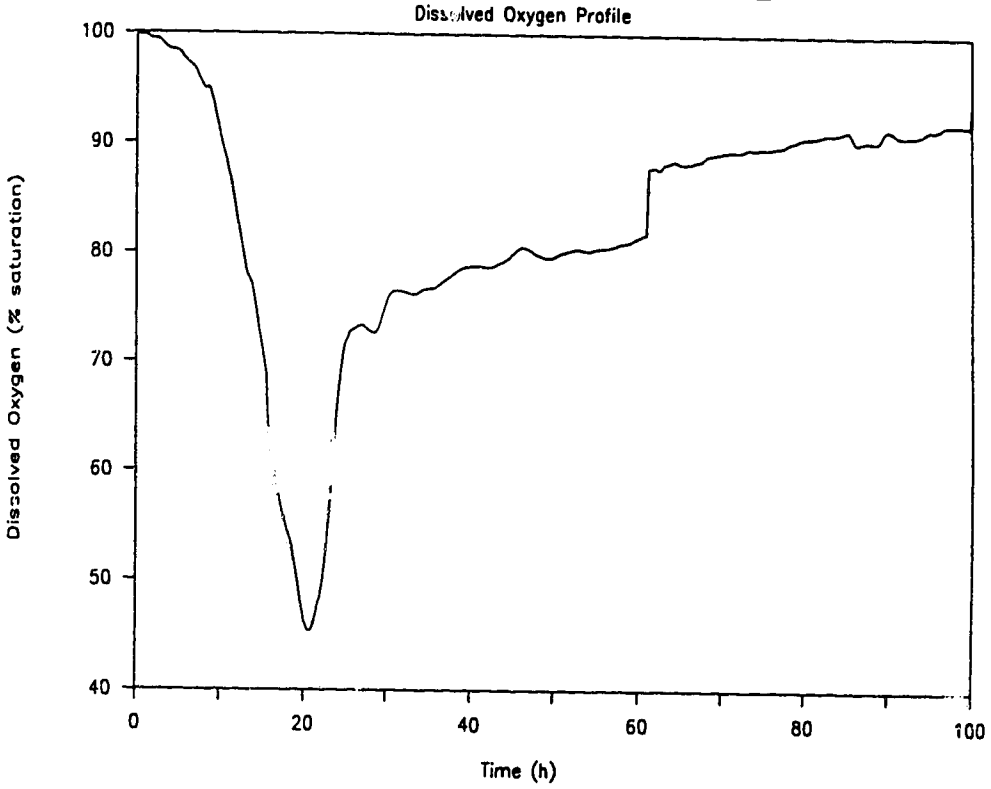


Figure C.4 Experiment Sclav_10

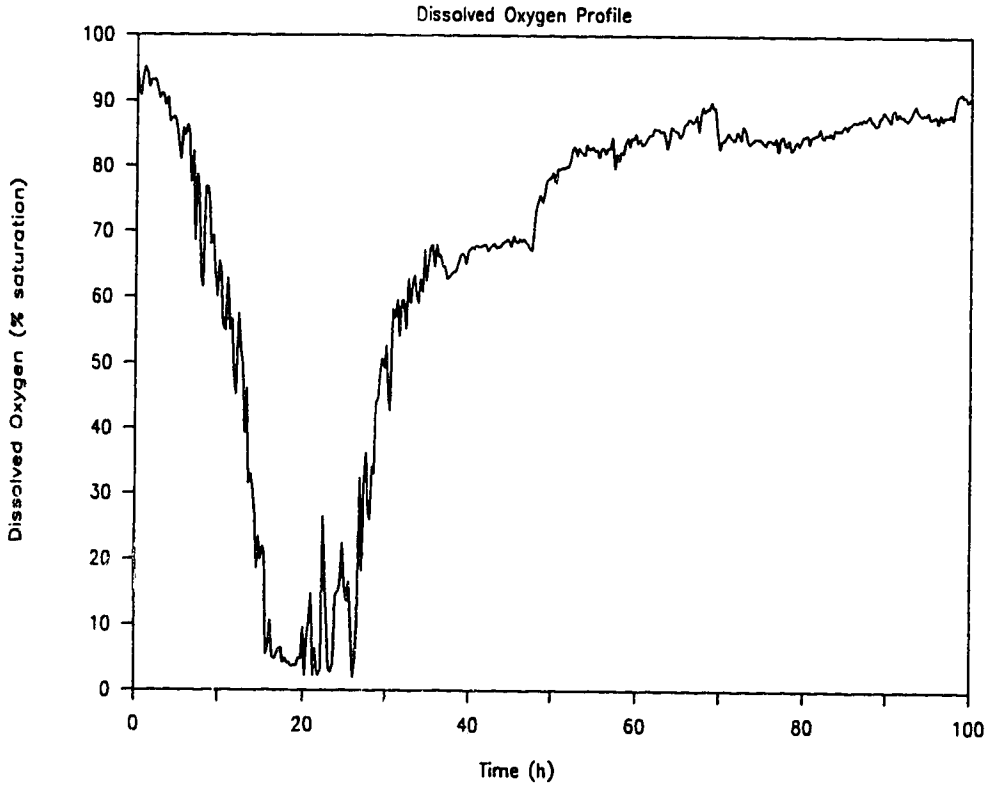


Figure C.5 Experiment Sclav_11

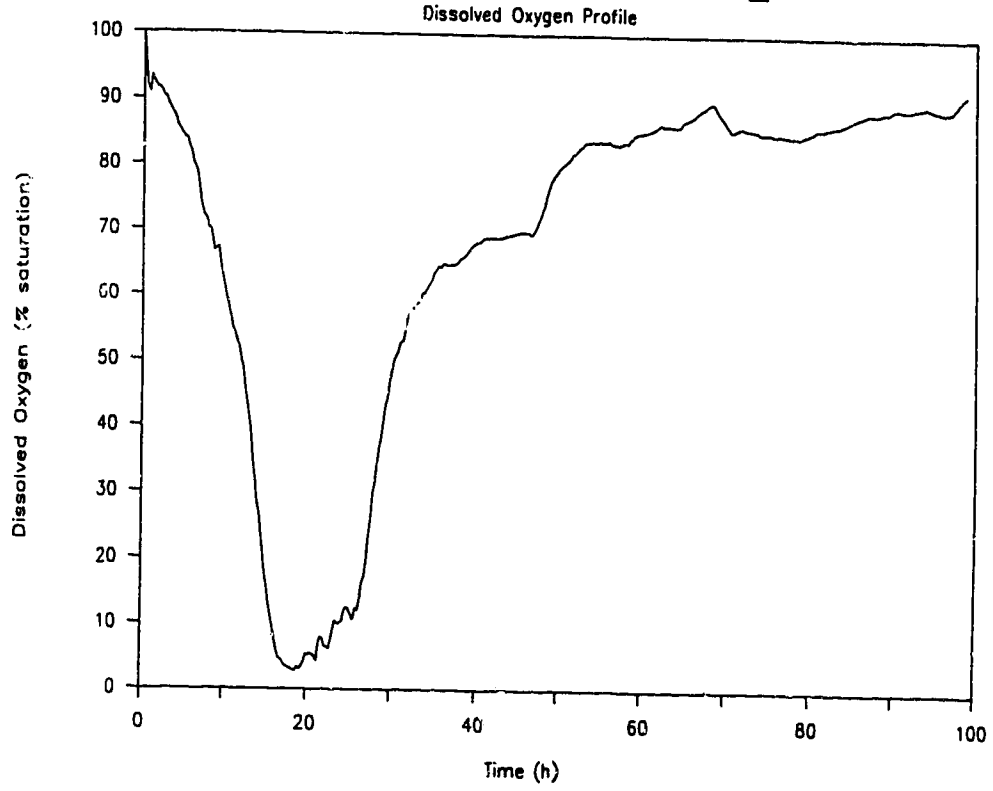


Figure C.6 Experiment Sclav_12

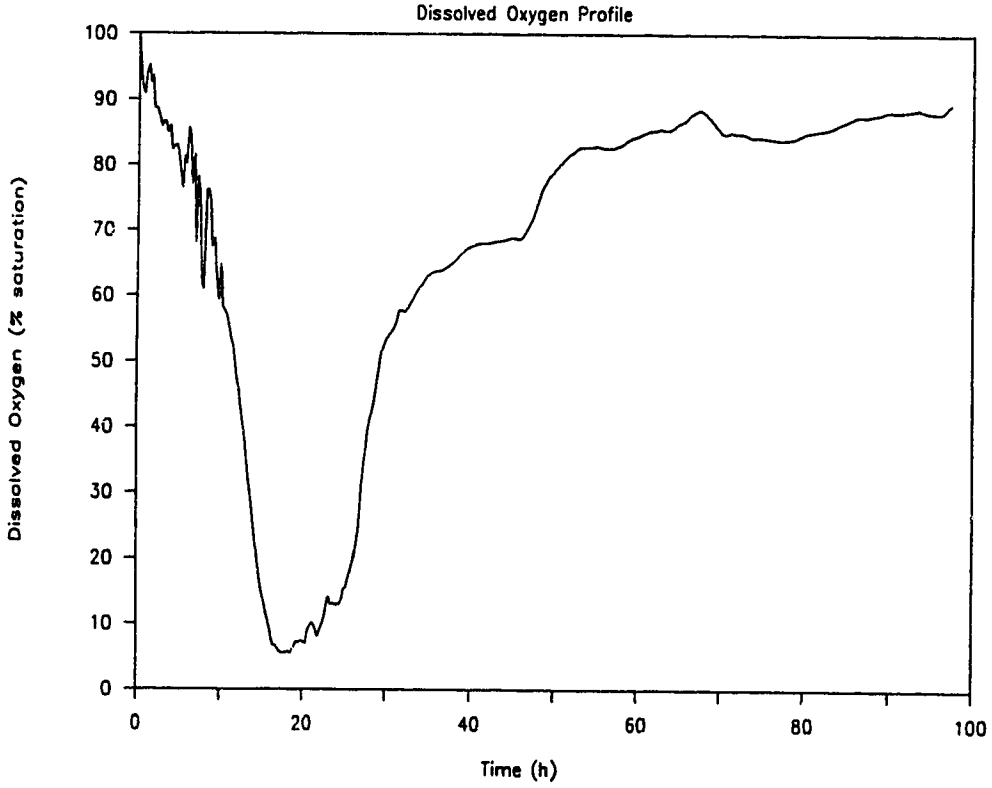


Figure C.7 Experiment Sclav_13

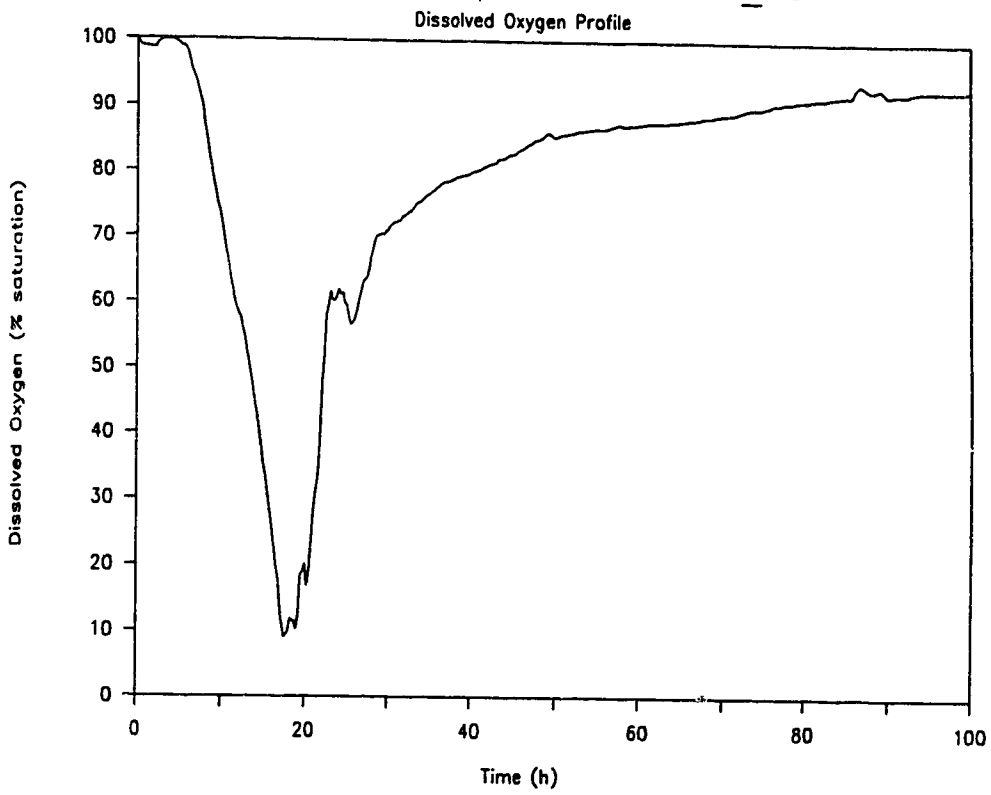


Figure C.8 Experiment Sclav_14

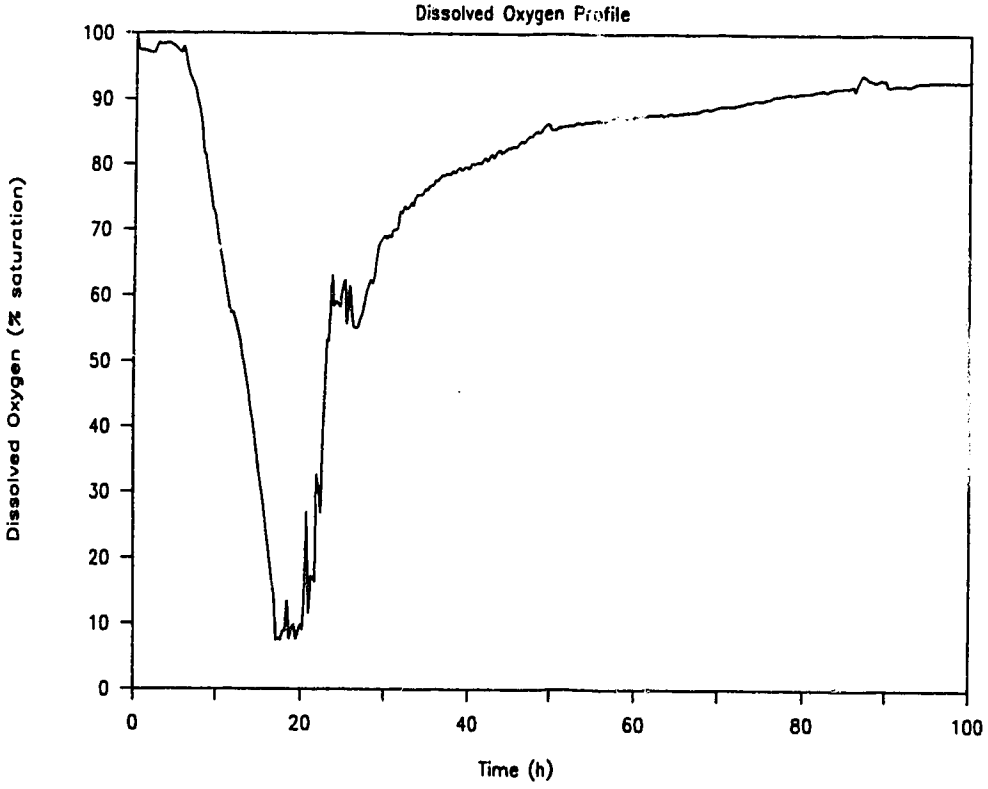
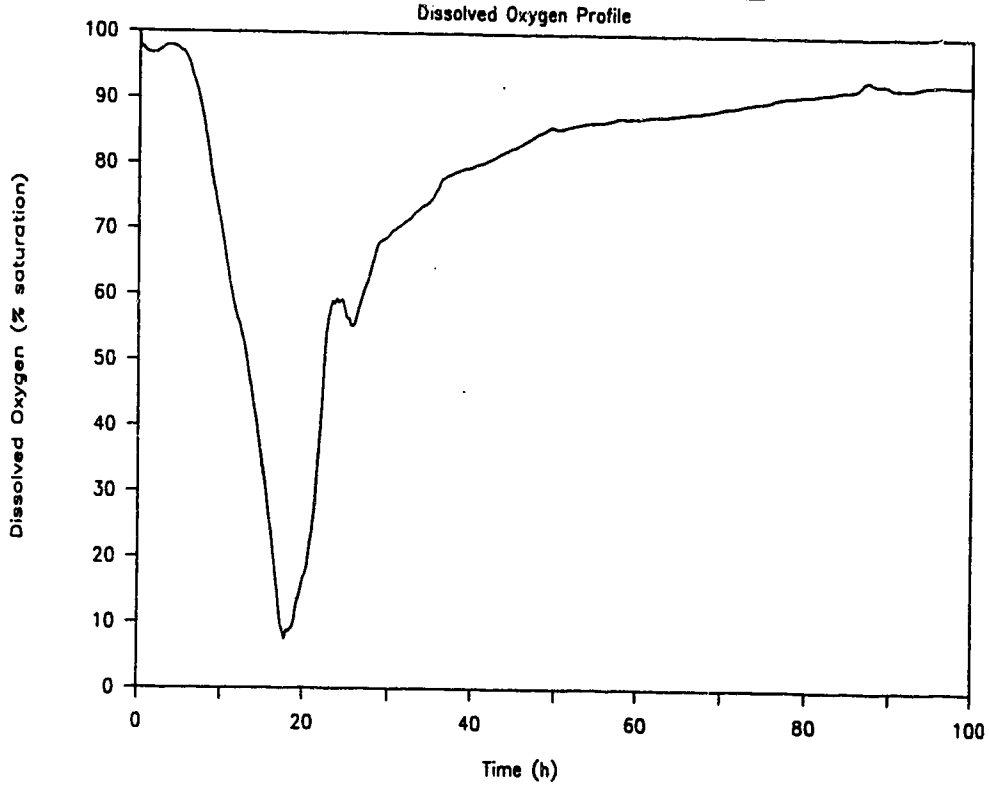


Figure C.9 Experiment Sclav_15



APPENDIX D

FERMENTOR DATA ACQUISITION AND CONTROL SYSTEM

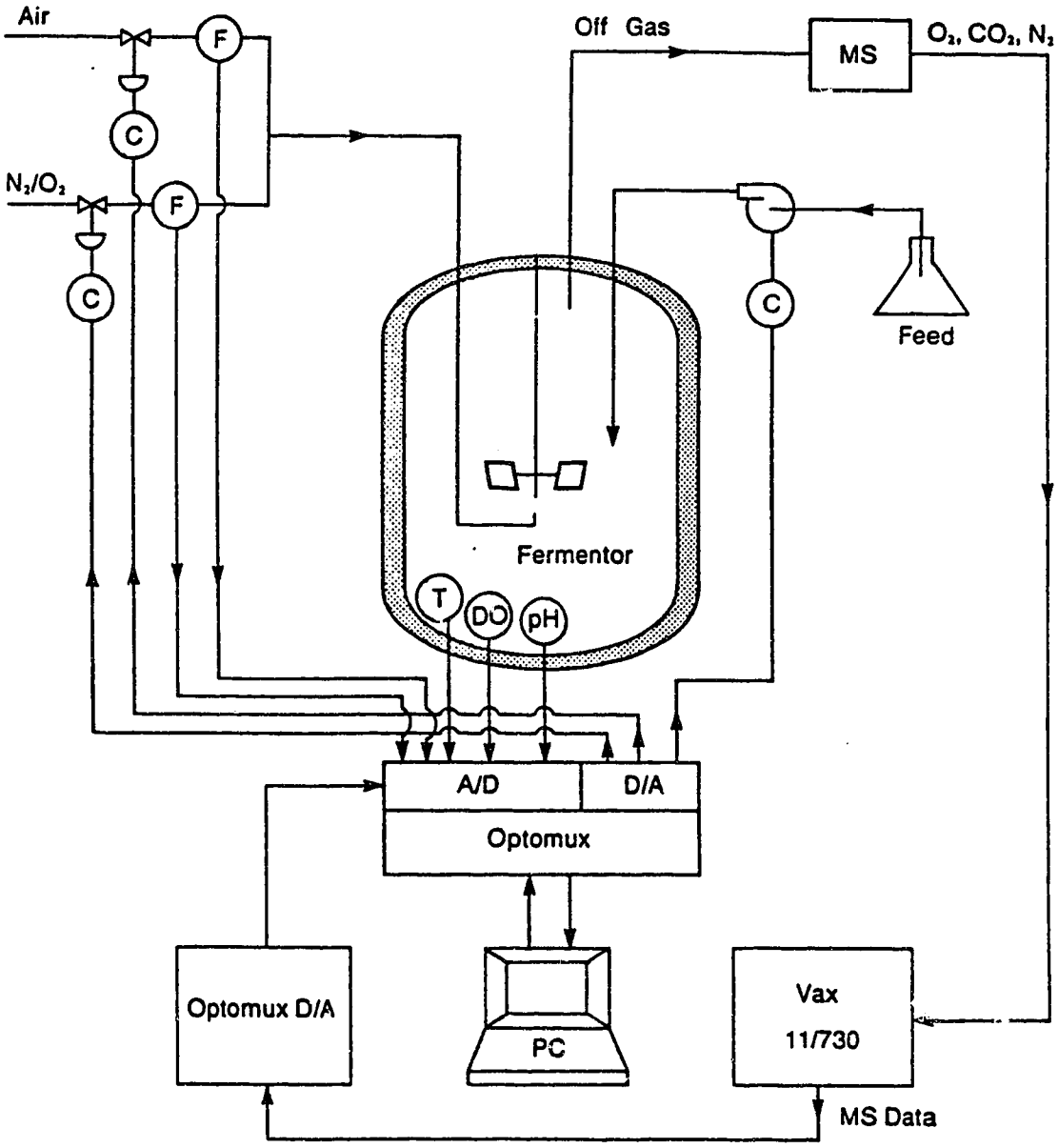


Figure D.1 Schematic diagram of the fermentor data acquisition and control system.

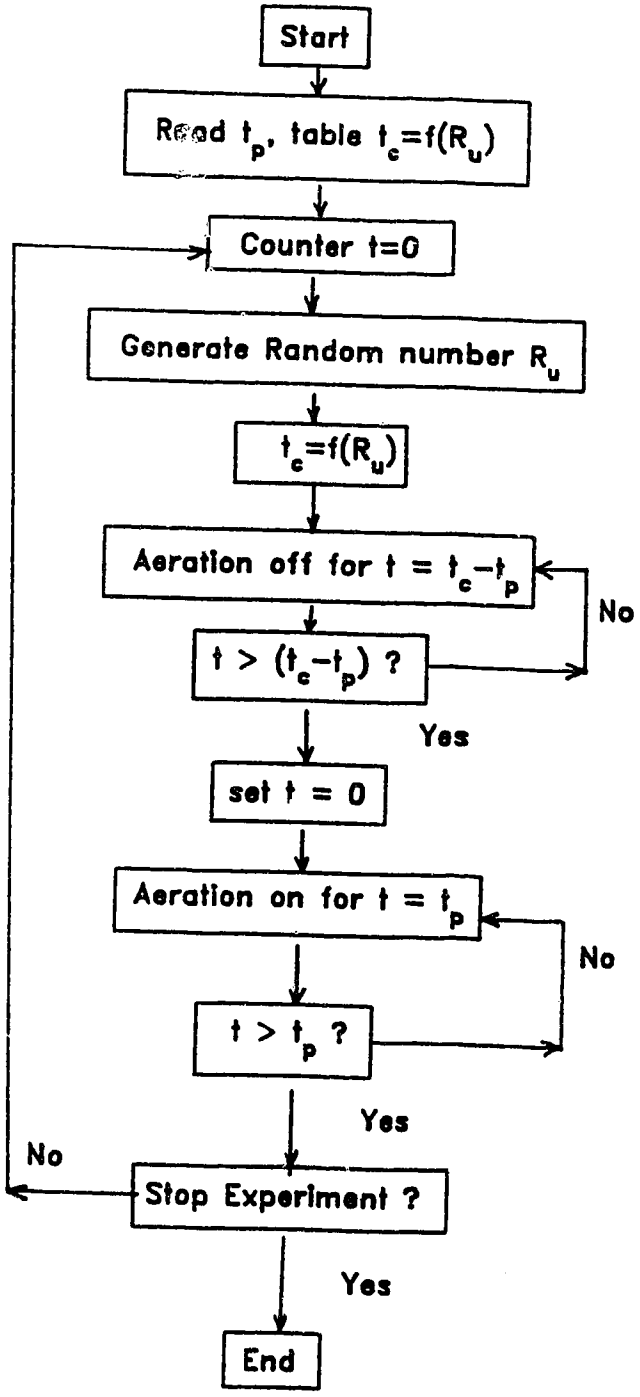


Figure D.2 Flow chart of software implementation for aeration control by the Monte Carlo method.