RECOGNITION OF PHASE TRANSITION IN FERMENTATION USING ON-LINE MONITORED VARIABLES.

A thesis submitted to the University of London for the degree of
DOCTOR OF PHILOSOPHY

by

Mitra DEHGHANI BEng.(Hons), MPhil

February 1996

Department of Chemical and Biochemical Engineering
University College London
Torrington Place
London WC1E 7JE
ABSTRACT

The aim of this research was to develop a technique by which aerobic batch stirred tank fermenters, containing the yeast *S. cerevisiae* (strain GB4918, Bakers yeast) cultured in a chemically defined and/or undefined medium with glucose as the initial limiting substrate at 30 g/L, pH 4.5 and temperature 28°C, as well as in repeated fill and draw batch bioreactors, can be controlled and operated by the use of a single on-line parameter that identifies and represents the complex biochemistry of the aerobic yeast and enables the batch cycle to be initiated and terminated, as well as the harvesting of intracellular enzymes of the yeast at optimum yield or concentration.

An extensive literature survey has been carried out into the aerobic culture of the yeast *S. cerevisiae* that identified key transition phases in the metabolism in relation to the operation of cellular metabolic pathways, (limited respiratory capacity, dioxic growth on ethanol).

Distinct changes in some of the on-line monitored variables of dissolved oxygen tension (DOT) and CO$_2$ exit gas concentration have been used to recognise the specific identifiable phases of glucose depletion, biomass formation and production of some intracellular enzymes, which in this project were alcohol dehydrogenase (ADH), glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), and hexokinase. Direct information about the metabolic activity of the cells and product concentration inside the batch bioreactor were obtained from the off-line samples taken from the culture broth. These on-line and off-line variables were correlated to enable process decision making to be based on calculated parameters rather than using a predetermined schedule.

An extensive data base has been set up from the experimentation in terms of the functioning of the yeast cells that gives the change in the external culture environment and the intracellular enzymes associated with glucose metabolism in an aerobic environment. These date place some considerable doubt on the published theories of the cell metabolic functioning.

The use of on-line CO$_2$ exit gas data has been clearly demonstrated as a means of assessing and regulating the operation of not only an experimental batch bioreactor, but also provides a reproducible and consistent means of controlling a commercial batch bioreactor.
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisors Mrs Nina Thornhill and Dr Andrew Ison for their advice, encouragement and support. I would also like to express my appreciation to Dr Malcolm Gregory, Dr Mark Bulmer and Dr Claire Turner for their help and their comments throughout the project.

Many sincere thanks to the technical staff of the Chemical and Biochemical Engineering department at University College London.

The support of the EPSRC and BBSRC through the Interdisciplinary Research Centre for Process Systems Engineering, Imperial College of Science, Technology and Medicine, and through Advanced Centre for Biochemical Engineering, University College London is gratefully acknowledged.

Finally, thanks to my family and my friends for their support and their encouragement.
# TABLE OF CONTENTS

Abstract 2  
Acknowledgments 3  
List of Figures 8  
List of Tables 18  
Nomenclature and abbreviations 19  
Thesis Outline 21

## Chapter 1  Introduction 22

1.1 Introduction to the project 22  
1.1.1 Choice of the micro-organism. 23  
1.1.2 Project aims and objective 24  
1.2 Yeast physiology 25  
1.2.1 Microbial yeast metabolism. 25  
1.2.2 Glucose metabolism in *S. cerevisiae* 27  
1.2.3 Regulation of glucose metabolism in *S. cerevisiae* 32  
1.3 Aerobic growth of *S. cerevisiae* in batch culture 37  
1.3.1 Repeated batch fermentations 39  
1.3.4 Fermentation products 42  
1.3.5 Inhibition of fermentation products 44  
1.4 Fermentation environmental variables 46  
1.4.1 Physical and chemical variables 46  
1.4.2 Measurement of fermentation variables 49  
1.4.3 Accuracy and reliability of measurement variables 51  
1.5 Fermentation measurements for control 53  
1.5.1 Direct fermentation variables 54  
1.5.1.1 Data bases and neural network 54  
1.5.2 Derived fermentation variables 55  
1.5.2.1 Fermentation phase profiling 56  
1.6 Summary 58

## Chapter 2  Materials and Methods 59

2.1 Fermentation 59  
2.1.1 Strain Selection and maintenance 59  
2.1.1.1 Microscopic examination of yeast 61  
2.1.2 Media and reagents 62  
2.1.2.1 YDP agar medium 62
3.3.2 CO₂ and DOT profiles

3.4 Summary

Chapter 4 Measurement Variability Analysis

4.1 Statistical methods
4.2 Production of broth samples for protein and enzyme assays
4.3 Off-line sample variability analysis
  4.3.1 Variability in biomass measurement
  4.3.2 Variability in soluble protein and ADH activity measurements
  4.3.3 Variability in replicate protein measurements
  4.3.4 Variability in replicate ADH measurements
  4.3.5 Variability in MDH, hexokinase, and G6PDH replicate measurements
  4.3.6 Variability in glucose, ethanol, and pyruvate replicate measurements
4.4 Summary

Chapter 5 Process Decision Making

5.1 Intracellular enzyme production schedules
5.2 Process automation
5.3 CO₂ peak detection
  5.3.1 Linearisation of CO₂ exit gas profile
  5.3.2 Derivative of the CO₂ profile
  5.3.3 On-line recognition of the CO₂ signal
5.4 Summary

6 Conclusions and Recommendations

6.1 Conclusions
6.2 Recommendations

Appendices

Appendix 1 Calibrating the turbidimetric method for Baker's yeast biomass determination.
Appendix 2  Experimental results from Single batches of *S. cerevisiae* fermentation with defined medium 'SBD'

Appendix 2.1 Fermentation results from SBD1

Appendix 2.2 Fermentation results from SBD2

Appendix 2.3 Fermentation results from SBD3

Appendix 3  Experimental results from a repeated batch culture of *S. cerevisiae* fermentation with defined medium 'RBD1'

Appendix 3.1 Fermentation results from RBD1-1

Appendix 3.2 Fermentation results from RBD1-2

Appendix 4  Experimental results from a repeated batch culture of *S. cerevisiae* fermentation with defined medium 'RBD2'

Appendix 4.1 Fermentation results from RBD2-1

Appendix 4.2 Fermentation results from RBD2-2

Appendix 4.3 Fermentation results from RBD2-3

Appendix 5  Experimental results from a repeated batch culture of *S. cerevisiae* fermentation with defined medium (RBD3)

Appendix 5.1 Fermentation results from RBD3-2

Appendix 5.2 Fermentation results from RBD3-3

Appendix 5.3 Fermentation results from RBD3-4

Appendix 6  Experimental results from a repeated batch culture of *S. cerevisiae* fermentation with complex medium (RBC2-2).

Appendix 7  LabView programming code

Appendix 7.1 LabView programming code to get CO$_2$ data

Appendix 7.2 LabView programming code to detect the first and the last CO$_2$ peak

References
LIST OF FIGURES

Chapter 1 Introduction
Figure 1.1 Aerobic metabolism of yeast adapted from Ratledge, (1991).
Figure 1.2 The breakdown of glucose to pyruvate with the simultaneous formation of ATP is referred to as Glycolytic (EMP) pathway, adapted from Stryer (1988).
Figure 1.3 Pathway of yeast intermediat metabolism. Pyruvate oxidises by the tri carboxylic cycle (TCA) to carbon dioxide and water.
Figure 1.4 Schematic representation of glucose metabolism in S. cerevisiae (adapted from Sonnleitner, 1991).
Figure 1.5 Limited respiratory capacity of S. cerevisiae illustrated as a bottleneck (Kappeli, 1986).
Figure 1.6 Regulation of glucose metabolism in S.cerevisiae based on limited respiratory capacity. Adapted from Kappeli, (1986)
Figure 1.7-a Aerobic growth of S.cerevisiae with glucose limiting substrate at an initial concentration of 20 g/L.Adapted from Fiechter et al., (1987)
Figure 1.7-b Oxygen uptake rate (mmolg\(^{-1}\)h\(^{-1}\)), carbon dioxide production rate (mmolg\(^{-1}\)h\(^{-1}\)), and respiratory quotient of an aerobic batch growth of S.cerevisiae. Adapted from Fiechter et al., (1987).
Figure 1.8 A graphical representation of 8 repetitive batch experiments of CO\(_2\) exhaust gas analysis.(Adapted from Locher et al., 1991, 1993).
Figure 1.9 Typical bioprocess interactions (Montague et al., 1989).

Chapter 2 Materials and Methods
Figure 2.1 Illustrates the acquisition of on-line and off-line data during the fermentations. (A process overview).
Figure 2.2 Diagram of the LH 7L fermenter used for all fermentations in this work.

Chapter 3 Fermentation data analysis- Results and discussion
Figure 3.1 CO\(_2\) exit gas profile of single batch 'SBD2' from inoculation time to the end of growth on ethanol.
Figure 3.2-a CO\(_2\) exit gas profile, and DOT profile of single batch culture 'SBD4' with defined medium.
Figure 3.2-b Off-line data of glucose (g/L), ethanol (g/L), and pyruvate (g/L) profile in single batch SBD4 with defined medium.

Figure 3.2-c Dry cell weight concentration (g/L) and total protein content (mg/mL) analysis of single batch fermentation SBD4.

Figure 3.2-d Dry cell weight concentration (g/L) and total protein content (g/mg biomass) analysis of single batch fermentation SBD4.

Figure 3.2-e Off-line assay of Alcohol dehydrogenase (U/mL) and Malate dehydrogenase (U/mL) in single batch fermentation.

Figure 3.2-f Glucose-6-phosphate dehydrogenase activity levels (U/mL) and Hexokinase activity levels (U/mL) in single batch fermentation SBD4.

Figure 3.2-g Off-line assay of Alcohol dehydrogenase (U/mL) and Malate dehydrogenase (U/mL) in single batch fermentation.

Figure 3.2-h Glucose-6-phosphate dehydrogenase activity levels (U/gDCW) and Hexokinase activity levels (U/gDCW) in SBD4.

Figure 3.2-i Off-line assay of Alcohol dehydrogenase (U/mg protein) and Malate dehydrogenase (U/mg protein) in single batch fermentation.

Figure 3.2-j Glucose-6-phosphate dehydrogenase activity levels (U/mg protein) and Hexokinase activity levels (U/mg protein) in SBD4.

Figure 3.3-a The log-log plot of ADH product formation in relation to biomass concentration from fermentation SBD4.

Figure 3.3-b The log-log plot of hexokinase product and biomass formation in SBD4.

Figure 3.3-c The log-log plot of MHD production and biomass formation during fermentation SBD4.

Figure 3.3-d The log-log plot of G6PDH product formation in relation to biomass concentration in SBD4 fermentation.

Figure 3.4-a Off-line data of glucose consumption (g/L) profile from single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-b Ethanol production profile (g/L) from single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-c Dry cell weight concentration (g/L) profiles from single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-d Optical density measurements of biomass concentration. Profiles in single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-e Total protein concentration profiles in single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.
Figure 3.4-f Total protein concentration profiles in single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-g ADH enzyme activity (U/mL) profiles from single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-h ADH enzyme activity (U/g DCW) profiles from single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-i ADH enzyme activity (U/mg protein) profiles from single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-j G6PDH enzyme activity (U/mL) profiles from single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-k G6PDH enzyme activity (U/g DCW) profiles from single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-l G6PDH enzyme activity (U/mg protein) profiles from single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-m MDH enzyme activity (U/mL) profiles from single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-n MDH enzyme activity (U/g DCW) profiles from single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-o MDH enzyme activity (U/mg protein) profiles from single batches with defined medium for SBD1, SBD2, SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-p Hexokinase enzyme activity (U/mL) profiles from single batches with defined medium for SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-q Hexokinase enzyme activity (U/g DCW) profiles from single batches with defined medium for SBD3, SBD4, and RBD2-1 batches.

Figure 3.4-r Hexokinase enzyme activity (U/mg protein) profiles from single batches with defined medium for SBD3, SBD4, and RBD2-1 batches.

Figure 3.5-a A graphical presentation of CO₂ exit gas profile in repeated batch culture (PRB1) on defined medium.

Figure 3.5-b DOT profile of the same experiment (PRB1) is presented.

Figure 3.6 DOT profile of repeated batch culture on defined medium RBD2.

Figure 3.6-a Off-line data of glucose consumption (g/L) profile from RBD2.

Figure 3.6-b Ethanol production profile (g/L) profile from RBD2.

Figure 3.6-c Dry cell weight concentration (g/L) profile from RBD2.

Figure 3.6-d Biomass optical density measurements. Profile from RBD2.

Figure 3.6-e Total protein concentration (U/mL) profile from RBD2.
Figure 3.6-f Total protein concentration (mg/mg DCW) profile from RBD2.
Figure 3.6-g ADH enzyme activity (U/mL) profile from RBD2.
Figure 3.6-h ADH enzyme activity (U/gDCW) profile from RBD2.
Figure 3.6-i ADH enzyme activity (U/mg protein) profile from RBD2.
Figure 3.6-j G6PDH enzyme activity (U/mL) profile from RBD2.
Figure 3.6-k G6PDH enzyme activity (U/mg DCW) profile from RBD2.
Figure 3.6-l G6PDH enzyme activity (U/mg protein) profile from RBD2.
Figure 3.6-m MDH enzyme activity (U/mL) profile from RBD2.
Figure 3.6-n MDH enzyme activity (U/g DCW) profile from RBD2.
Figure 3.6-o MDH enzyme activity (U/mg protein) profile from RBD2.
Figure 3.6-p Hexokinase enzyme activity (U/mL) profile from RBD2.
Figure 3.6-q Hexokinase enzyme activity (U/g DCW) profile from RBD2.
Figure 3.6-r Hexokinase enzyme activity (U/mg protein) profile from RBD2.
Figure 3.6-s Pyruvate (g/L) profile from RBD2.

Figure 3.7-a A graphical presentation of normalised CO2 exit gas profile in repeated batch culture RBD3.
Figure 3.7-b A graphical presentation of DOT exit gas profile in repeated batch culture RBD3.

Figure 3.8-a Off-line data of glucose consumption (g/L) profile from RBD3.
Figure 3.8-b Ethanol production profile (g/L), profile from RBD3.
Figure 3.8-c Dry cell weight concentration (g/L) profile from RBD3.
Figure 3.8-d Biomass optical density measurements. profile from RBD3.
Figure 3.8-e Total protein concentration (U/mL) profile from RBD3.
Figure 3.8-f Total protein concentration (U/mg DCW) profile from RBD3.
Figure 3.8-g ADH enzyme activity (U/mL) profile from RBD3.
Figure 3.8-h ADH enzyme activity (U/gDCW) profile from RBD3.
Figure 3.8-i ADH enzyme activity (U/mg protein) profile from RBD3.
Figure 3.8-j G6PDH enzyme activity (U/mL) profile from RBD3.
Figure 3.8-k G6PDH enzyme activity (U/g DCW) profile from RBD3.
Figure 3.8-l G6PDH enzyme activity (U/mg protein) profile from RBD3.
Figure 3.8-m MDH enzyme activity (U/mL) profile from RBD3.
Figure 3.8-n MDH enzyme activity (U/g DCW) profile from RBD3.
Figure 3.8-o MDH enzyme activity (U/mg protein) profile from RBD3.
Figure 3.8-p Hexokinase enzyme activity (U/mL) profile from RBD3.
Figure 3.8-q Hexokinase enzyme activity (U/mg DCW) profile from RBD3.
Figure 3.8-r Hexokinase enzyme activity (U/mg protein) profile from RBD3.
Figure 3.8-s Pyruvate concentration (g/L) profile from RBD3.
Figure 3.9-a CO\textsubscript{2} exit gas profile, and DOT profile of 'RBC1-2'.

Figure 3.9-b Off-line data of glucose, ethanol, and pyruvate (g/L) in RBC1-2.

Figure 3.9-c Dry cell weight concentration (mg/mL) and total soluble protein content (mg/mL) analysis of RBC1-2.

Figure 3.9-d Dry cell weight concentration (g/L) and total soluble protein content (g/gDCW) analysis of RBC1-2.

Figure 3.9-e Off-line assay of Alcohol dehydrogenase (U/mL) and Malate dehydrogenase (U/mL) in RBC1-2.

Figure 3.9-f Glucose-6-phosphate dehydrogenase activity levels (U/mL) and hexokinase enzyme activity level (U/mL) in RBC1-2.

Figure 3.9-g Off-line assay of Alcohol dehydrogenase (U/gDCW) and Malate dehydrogenase (U/gDCW) RBC1-2.

Figure 3.9-h Glucose-6-phosphate dehydrogenase activity levels (U/gDCW) and hexokinase enzyme activity level (U/gDCW) in RBC1-2.

Figure 3.9-i Off-line assay of Alcohol dehydrogenase (U/mg protein) and Malate dehydrogenase (U/mg protein) in RBC1-2.

Figure 3.9-j Glucose-6-phosphate dehydrogenase activity levels (U/mg protein) and hexokinase enzyme activity level (U/mg protein) in RBC1-2.

Figure 3.10-a CO\textsubscript{2} exit gas profile in repeated batch culture (RBC2) with complex medium.

Figure 3.10-b DOT exit gas profile in repeated batch culture (RBC2) with complex medium.

Chapter 4 Measurement Variability Analysis

Figure 4.1 Statistics of the biomass optical density measurements (660 nm).

Figure 4.2-a Statistics of soluble protein assay.

Figure 4.2-b The log-log plot of statistics of soluble protein assay.

Figure 4.3-a Replicate measurements of ADH activity from a single broth sample.

Figure 4.3-b Statistics of ADH assay.

Figure 4.4 Statistics of additional intracellular enzyme assays.

Figure 4.5 Statistics of HPLC analysis.

Chapter 5 Process Decision Making

Figure 5.1 CO\textsubscript{2} exit gas analysis profile and the ADH enzyme activity (U/mL) from SBD4.
Figure 5.2  Piecewise linearisation of CO\textsubscript{2} exit gas data from fermentation SBD2, using the software developed by Marshall (1992).

Figure 5.3  CO\textsubscript{2} exit gas profile from SBD2 fermentation with defined medium using Matlab programming environment.

Figure 5.4  CO\textsubscript{2} exit gas profile from RBC2-1 fermentation with complex medium using Matlab programming environment.

Figure 5.5  CO\textsubscript{2} exit gas profile from SBD2 fermentation with defined medium using LabVIEW programming environment.

Chapter 6  Conclusions and recommendations

Figure 6.1  CO\textsubscript{2} exit gas analysis profile and RQ plot for SBD2.

Appendix 1

Figure A1.1  Calibration curve of absorbance verses yeast cell concentration.
Figure A1.2  Calibration curve of absorbance verses yeast cell concentration. All samples are diluted using DW.
Figure A1.3  Calibration curve of absorbance verses yeast cell concentration.

Appendix 2

Figure A2.1-a  CO\textsubscript{2} exit gas profile and DOT profile of single batch culture 'SBD1' on define medium.
Figure A2.1-b  Off-line data of glucose, ethanol, and pyruvate profiles in single batch SBD1
Figure A2.1-c  Dry cell weight concentration and total protein content analysis of single batch fermentation SBD1 in define medium.
Figure A2.1-f  Off-line assay of Alcohol dehydrogenase (U/ml) and Malate dehydrogenase (U/ml) in single batch fermentation.
Figure A2.1-g  Glucose-6-phosphate dehydrogenase activity levels (U/mL) in single batch fermentation SBD1.

Figure A2.2-a  CO\textsubscript{2} exit gas profile and DOT profile of single batch culture 'SBD2' on define medium.
Figure A2.2-b  Off-line data of glucose, ethanol, and pyruvate profiles in single batch SBD2 with define medium.
Figure A2.2-c  Dry cell weight concentration and total protein content analysis of single batch fermentation SBD2 in define medium.
Figure A2.2-d Off-line assay of Alcohol dehydrogenase (U/mL) and Malate dehydrogenase (U/mL) in single batch fermentation.

Figure A2.2-e Glucose-6-phosphate dehydrogenase activity levels (U/mL) in single batch fermentation SBD2

Figure A2.3-a CO₂ exit gas profile and DOT profile of single batch culture 'SBD3' on define medium.

Figure A2.3-b Off-line data of glucose, ethanol, and pyruvate profiles in single batch SBD3 with define medium.

Figure A2.3-c Dry cell weight concentration and total protein content analysis of single batch fermentation SBD3 in define medium.

Figure A2.3-d Off-line assay of Alcohol dehydrogenase and MDH activity levels (U/mL) in single batch fermentation.

Figure A2.3-e Glucose-6-phosphate dehydrogenase activity levels (U/mL) and hexokinase enzyme activity level (U/mL) in single batch fermentation SBD3

Appendix 3

Figure A3.1-a CO₂ exit gas profile and DOT profile of repeated batch culture 'RBD1-1' on defined medium.

Figure A3.1-b Off-line data of glucose, ethanol profile in repeated batch RBD1-1 with defined medium.

Figure A3.1-c Dry cell weight concentration and total soluble protein content analysis of repeated batch fermentation RBD1-1 in defined medium.

Figure A3.1-d Off-line assay of Alcohol dehydrogenase (U/mL) and Malate dehydrogenase (U/mL) in repeated batch fermentation RBD1-1.

Figure A3.1-e Glucose-6-phosphate dehydrogenase activity levels (U/mL) in repeated batch fermentation RBD1-1.

Figure A3.2-a CO₂ exit gas profile of repeated batch culture 'RBD1-2' on defined medium.

Figure A3.2-b Off-line data of glucose, ethanol profile in repeated batch RBD1-2 with defined medium.

Figure A3.2-c Dry cell weight concentration and total soluble protein content analysis of repeated batch fermentation RBD1-2 in defined medium.
Appendix 4

Figure A4.1-a  DOT profile of repeated batch culture 'RBD2-1' on defined medium.

Figure A4.1-b  Off-line data of glucose, ethanol, and pyruvate profiles in repeated batch RBD2-1 with defined medium.

Figure A4.1-c  Dry cell weight concentration and total soluble protein content analysis of repeated batch fermentation RBD2-1 in defined medium.

Figure A4.1-d  Off-line assay of Alcohol dehydrogenase (U/mL) and Malate dehydrogenase (U/mL) in repeated batch fermentation.

Figure A4.1-e  Glucose-6-phosphate dehydrogenase activity levels (U/mL) and Hexokinase activity levels (U/mL) in repeated batch fermentation RBD2-1.

Figure A4.2-a  DOT profile of repeated batch culture 'RBD2-2' on defined medium.

Figure A4.2-b  Off-line data of glucose, ethanol and pyruvate profiles in repeated batch RBD2-2 with defined medium.

Figure A4.2-c  Dry cell weight concentration and total soluble protein content analysis of repeated batch fermentation RBD2-2 in defined medium.

Figure A4.2-d  Off-line assay of Alcohol dehydrogenase (U/mL) and Malate dehydrogenase (U/mL) in repeated batch fermentation.

Figure A4.2-e  Glucose-6-phosphate dehydrogenase activity levels (U/mL) and Hexokinase activity levels (U/mL) in repeated batch fermentation RBD2-2.

Figure A4.3-a  DOT profile of repeated batch culture 'RBD2-3' on defined medium.

Figure A4.3-b  Off-line data of glucose, ethanol, and pyruvate profiles in repeated batch RBD2-3 with defined medium.
Figure A4.3-c  Dry cell weight concentration and total soluble protein content analysis of repeated batch fermentation RBD2-3 in defined medium.

Figure A4.3-d Off-line assay of Alcohol dehydrogenase (U/mL) and Malate dehydrogenase (U/mL) in repeated batch fermentation.

Figure A4.3-e Glucose-6-phosphate dehydrogenase activity levels (U/mL) and Hexokinase activity levels (U/mL) in repeated batch fermentation RBD2-3.

Appendix 5
Figure A5.1-a CO₂ exit gas profile and DOT profile of repeated batch culture 'RBD3-2' on defined medium.

Figure A5.1-b Off-line data of glucose, ethanol, and pyruvate profiles in repeated batch RBD3-2 with defined medium.

Figure A5.1-c Dry cell weight concentration and total soluble protein content analysis of repeated batch fermentation RBD3-2 in defined medium.

Figure A5.1-d Off-line assay of Alcohol dehydrogenase (U/mL) and Malate dehydrogenase (U/mL) in repeated batch fermentation.

Figure A5.1-e Glucose-6-phosphate dehydrogenase activity levels (U/mL) and Hexokinase activity levels (U/mL) in repeated batch fermentation RBD3-2.

Figure A5.2-a CO₂ exit gas profile and DOT profile of repeated batch culture 'RBD3-3' on defined medium.

Figure A5.2-b Off-line data of glucose, ethanol, and pyruvate profiles in repeated batch RBD3-3 with defined medium.

Figure A5.2-c Dry cell weight concentration and total soluble protein content analysis of repeated batch fermentation RBD3-3 in defined medium.

Figure A5.2-d Off-line assay of Alcohol dehydrogenase (U/mL) and Malate dehydrogenase (U/mL) in repeated batch fermentation.

Figure A5.2-e Glucose-6-phosphate dehydrogenase activity levels (U/mL) and Hexokinase activity levels (U/mL) in repeated batch fermentation RBD3-3.

Figure A5.3-a CO₂ exit gas profile and DOT profile of repeated batch culture 'RBD3-4' on defined medium.

Figure A5.3-b Off-line data of glucose, ethanol, and pyruvate profiles in repeated batch RBD3-4 with defined medium.
Figure A5.3-c  Dry cell weight concentration and total protein content analysis of repeated batch fermentation RBD3-4 in defined medium.

Figure A5.3-d  Off-line assay of Alcohol dehydrogenase (U/mL) and Malate dehydrogenase (U/mL) in repeated batch fermentation RBD3-4.

Figure A5.3-e  Glucose-6-phosphate dehydrogenase activity levels (U/mL) and Hexokinase activity levels (U/mL) in repeated batch fermentation RBD3-4.

Appendix 6

Figure A6.1-a  CO₂ exit gas profile and DOT profile of repeated batch culture 'RBC2-2' on complex medium.

Figure A6.1-b  Off-line data of glucose, and pyruvate profiles in repeated batch RBC2-2 with complex medium.

Figure A6.1-c  Dry cell weight concentration and total soluble protein content analysis of repeated batch fermentation RBC2-2 in complex medium.

Figure A6.1-d  Off-line assay of Alcohol dehydrogenase (U/mL) and Malate dehydrogenase (U/mL) in repeated batch fermentation RBC2-2.

Figure A6.1-e  Glucose-6-phosphate dehydrogenase activity levels (U/mL) and Hexokinase enzyme activity level (U/mL) in single batch fermentation RBC2-2.

Appendix 7

Figure A7.1  LabVIEW visual program code to get CO₂ data.

Figure A7.2  LabVIEW visual program code to detect the first and the last CO₂ peaks.
LIST OF TABLES

Table 1.1 Changes in the activity of enzymes during growth of yeast (Adapted from Polakis and Bartley, 1965).
Table 1.2 Effect of ethanol on specific enzyme activities of yeast S.cerevisiae in cell extract and whole cells (Adapted from Pascual et al., 1988).
Table 1.4 Performance of measurement and control instrumentation in a 1 m$^3$ pilot plant bioreactor (Flynn, 1982).
Table 1.5 Integration of computer capabilities (Adapted from Omstead, 1991).
Table 2.1 The solid media used for storage of Saccharomyces cerevisiae. (Fiechter et al., 1981).
Table 2.2 The total concentration of defined medium used for cultivation of Saccharomyces cerevisiae. (Fiechter et al., 1981).
Table 2.3 The defined media used for cultivation of S.cerevisiae (Fiechter et al., 1981).
Table 2.4 The composition of the MYGP culture medium.
Table 2.5 Set points of the controlled variables for all fermentations.
Table 2.6 Measured and monitored variables during the time course of the fermentation processes.
Table 3.1 Fermentation experiments using defined medium.
Table 3.2 A matrix of fermentation experiments using defined medium. Emboldened experiments are included in SBD comparison figures.
Table 3.3 A matrix of fermentation experiments using defined medium. Emboldened experiments are included in RBD section.
Table 3.4 Estimated fermentation time at which the first CO$_2$ peak had occurred for RBD2-1,3 from on-line monitored DOT data.
Table 4.1 Standard deviation of some assays for S.cerevisiae.

Table A1.1 The composition of the solution made (g/L).
Table A1.2 Replicate measurements of optical density and dry cell weight concentration for several measurement sets.
Table A1.3 At concentrations 0.3 g/L and 0.8 g/L, 5 more replicate of the measurements were performed using solution S to suspend the yeast.
**NOMENCLATURE and ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine tri-phosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine di-phosphate</td>
</tr>
<tr>
<td>CER</td>
<td>carbon dioxide evolution rate</td>
</tr>
<tr>
<td>c</td>
<td>the molar concentration of all particles within the sample</td>
</tr>
<tr>
<td>(CO₂)₀</td>
<td>calculated center point of the CO₂ interval</td>
</tr>
<tr>
<td>(CO₂)ᵢ</td>
<td>ith value of the calculated center point of the CO₂ interval</td>
</tr>
<tr>
<td>(CO₂)ᵢᵢ</td>
<td>ith value of the CO₂ data in the interval</td>
</tr>
<tr>
<td>d</td>
<td>thickness of the substance</td>
</tr>
<tr>
<td>D</td>
<td>dilution factor</td>
</tr>
<tr>
<td>DO</td>
<td>dissolved oxygen</td>
</tr>
<tr>
<td>DOT</td>
<td>dissolved oxygen tension (%saturation)</td>
</tr>
<tr>
<td>DCW</td>
<td>dry cell weight</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EMP</td>
<td>Emdben-Meyerjof-Parnas</td>
</tr>
<tr>
<td>G6PDH</td>
<td>glucose-6- phosphate dehydrogenase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>I₀</td>
<td>intensity of light passed through a substance</td>
</tr>
<tr>
<td>I</td>
<td>the intensity of transmitted light</td>
</tr>
<tr>
<td>IMS</td>
<td>industrial methylated spirits</td>
</tr>
<tr>
<td>Kᵢ</td>
<td>the calibration constant</td>
</tr>
<tr>
<td>MAX</td>
<td>maximum</td>
</tr>
<tr>
<td>MDH</td>
<td>malate dehydrogenase</td>
</tr>
<tr>
<td>n</td>
<td>number of replicate measurements</td>
</tr>
<tr>
<td>N</td>
<td>number of sequential data</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>reduced form of nicotinamide adenine dinucleotide (phosphate)</td>
</tr>
<tr>
<td>NAD(P)</td>
<td>nicotinamide adenine dinucleotide (phosphate)</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OUR</td>
<td>oxygen uptake rate</td>
</tr>
<tr>
<td>PHB</td>
<td>poly-β-hydroxybutyrate</td>
</tr>
<tr>
<td>PID</td>
<td>proportional, differential, integral (control)</td>
</tr>
<tr>
<td>PRB</td>
<td>preliminary repeated batch culture</td>
</tr>
<tr>
<td>RI</td>
<td>refractive index</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>RBC</td>
<td>repeated batch fermentation on complex medium</td>
</tr>
<tr>
<td>RBD</td>
<td>repeated batch fermentation on defined medium</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>RQ</td>
<td>respiratory quotient</td>
</tr>
<tr>
<td>$s^2$</td>
<td>sample variance</td>
</tr>
<tr>
<td>$s$</td>
<td>standard deviation of the replicates</td>
</tr>
<tr>
<td>SBD</td>
<td>single batch fermentation on defined medium</td>
</tr>
<tr>
<td>$(t)_{ci}$</td>
<td>ith value of the calculated center point of the time interval</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetate acid</td>
</tr>
<tr>
<td>TCS</td>
<td>Turnbull control system</td>
</tr>
<tr>
<td>$T_{cmax}$</td>
<td>time at which maximum CO$_2$ occurs</td>
</tr>
<tr>
<td>$T_{ferm}$</td>
<td>actual fermentation time</td>
</tr>
<tr>
<td>$T_{rel}$</td>
<td>relative fermentation time to the first CO$_2$ peak</td>
</tr>
<tr>
<td>tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>$V_t$</td>
<td>total volume of the assay mixture</td>
</tr>
<tr>
<td>$V_s$</td>
<td>sample size in the cuvette</td>
</tr>
<tr>
<td>VI</td>
<td>virtual instruments</td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>an estimate of the population mean</td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>the sample mean</td>
</tr>
<tr>
<td>$x_i$</td>
<td>the replicate measurements at a particular concentration</td>
</tr>
<tr>
<td>$\sigma^2$</td>
<td>population variance</td>
</tr>
<tr>
<td>$\Delta A$</td>
<td>rate of change of absorbence</td>
</tr>
<tr>
<td>$\Delta T$</td>
<td>time interval</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>the molar extinction coefficient</td>
</tr>
</tbody>
</table>
THESIS OUTLINE

Chapter 1 includes the introduction to the thesis and a literature review carried out into yeast physiology, aerobic growth of *S. cerevisiae* in batch culture, effect of environmental variables on fermentations and fermentation measurements for control. The brief literature survey is given to provide the basis for understanding the complexity of bioprocesses and the limitations in measurements for control of these processes.

The experimental methodology and instrumentation used in this project are reported in chapter 2.

Fermentation data analysis and basic results are discussed in chapter (3) and the associated appendices where on-line and off-line data correlations from defined medium fermentations are investigated, reproducibility of the correlations are tested and profiles of the same variables from complex medium fermentations are presented as a comparison.

Chapter (4) indicates the variability analysis of the off-line sample measurements carried out for this project. Summary statistics of replicate sets of measurements from fermentation broth samples are indicated.

The mathematical technique for on-line phase transition recognition is described in chapter (5). Signals from the CO₂ exit gas profiles are identified to base the process decision making on the first and the last peak of the CO₂ profile.

Conclusions and recommendations are reported in chapter 6.
1 INTRODUCTION

This chapter includes an introduction to the project (section 1.1) and a literature review (sections 1.2-1.5) into yeast physiology, aerobic batch fermentation of *S. cerevisiae*, fermentation environmental variables, and the application of fermentation variables to the control of these processes, to provide the basis for understanding the application of on-line monitored variables for process decision making.

1.1 Introduction to the project

Complexity of bioprocesses and the multitude of control variables has led to a wide range of possibilities for optimisation of these processes. Wilson (1991), has reviewed the basic techniques available for bioprocess control, and identified the main limitation and extensions of these techniques.

Monitoring and control of fermentation processes are necessary to ensure consistent performance of these processes (Salmon and Buckland, 1992). Many industrial processes are run using reliable measured parameter changes as a basis for process decision making (such as inoculation, induction, and feeding). Most parameters that can be directly determined are measurement of the physical conditions of the bioreactor. Sampling of the broth is required for analysis of many compounds that give information about the state of the fermentation. Off-line handling of the broth samples are often time consuming and involve an interruption of the process.

Although recent progress in instrumentation and measurement technology of the bioreactors has helped the ability to obtain qualitative knowledge about these processes and their behaviour, the problem of availability of measurement sensors for some essential variables constrains the control options of the processes (Royce 1993).
Availability of on-line measurements to recognise the specific identifiable events such as nutrient depletion and product formation for further process decision making (timing of the feed, harvesting a specific product, and automation of refilling the bioreactor) is an issue to be considered. Reliability of these measurements must also be considered, specially when the cost of the raw material is significance for some large scale fermentation operations.

Other factors which that need to be taken into account in attempting to use the monitored variables for process decision making are:

* Possibility of obtaining indirect information about the state of the fermentation by correlating the off-line sample measurements and the monitored variables.

* Reproducibility of the correlations of the two sets of monitored and measured data.

* Reliability of the monitored and measured variables.

* Availability of a signal form profiles of the on-line variables.

* Simplicity of detecting the signal.

1.1.1 Choice of the micro-organism.

The selected micro-organism was *Saccharomyces cerevisiae* strain (GB4918 provided by Distiller’s Company Ltd.) a Bakers yeast. *S. cerevisiae* was chosen as this micro-organism satisfied the following criteria;

a) *S. cerevisiae* is commercially an important micro-organism in brewing, baking, and food manufacture.

b) There has been much research on microbiology, biochemistry, modelling and kinetics of yeast growth.

c) Yeast has an advantage over many organisms in being able to grow easily under both aerobic and anaerobic conditions, on defined and complex medium, and on a range of substrates.

d) Ease of cultivation makes the microbe suitable for quick data generation.

e) *S. cerevisiae* has been successfully cultured aerobically at pH 4.5 in a chemically defined medium, where the low acidic pH helps prevent contamination infections.

f) Gene cloning has been applied to this micro-organism for higher and improved product quality.
1.1.2 Project aims and objective

With reference to the problems mentioned about the measurement for control of fermentations in section 1.1, the objective of this project was:

To use easily monitored variables (CO\textsubscript{2} and DOT profiles) and their correlations with the biological events inside the batch bioreactor (nutrient depletion, and product formation) to aid process decision making (e.g. the timing of seed transfer, prediction of maximum product formation) for a better process operation and control of the batch bioreactor.

Accordingly, in order to achieve this objective, the following aims were identified:

a) To investigate the enzyme production levels during the exponential growth phase, through the transition into the stationary phase of the yeast growth. Intracellular enzymes such as G6PDH, hexokinase, ADH, and MDH were chosen as metabolic indicators of the transition phase.

b) To identify the correlations between the on-line monitored variables and the intracellular enzyme activity levels measured off-line, and to indicate the reproducibility of the correlations between these variables.

c) To study the reproducibility of the assay profiles and to reduce the batch to batch variability of the profiles by performing repeated batch cultures under the same fermentation conditions as the batch cultures.

d) To assess the variability of the off-line measurements to obtain a knowledge of the measurement error for establishing the validity of the correlations and events observed in the fermentations.

e) To use mathematical techniques to identify a signal from the on-line monitored variables, indicating the transition of the fermentation phase, where process decisions such as timing of the harvest or refilling the tank could be based on the recognised signal.

The thesis describes the methods and results for these objectives, and demonstrates the achievements of all the above objectives.
1.2 Yeast physiology

Saccharomyces cerevisiae physiology has been reviewed by many authors including Rose and Harrison (1987, 1989), Fraenkel (1982), Berry and Brown (1987), and Ratledge (1991). An overview of microbial yeast metabolism, glucose metabolism, and regulation of glucose metabolism is presented in this section. The information is used as a reference to understanding of glucose substrate uptake and cellular metabolic activity for production of some intracellular enzymes investigated in this work.

1.2.1 Microbial yeast metabolism

Ratledge (1991) defined the metabolism as the overall biochemical reactions that take place inside the living organism for transformation of nutrients to cellular material. Metabolism is a combination of catabolism (degradation) and anabolism (biosynthesis) processes shown in Figure 1.1.

Catabolism is the break down of complex organic compounds by the cells to give a number of carbon intermediates as building blocks for cellular material, as well as providing energy in the form of ATP and some reducing power (NADH and NADPH). Whilst, anabolism is the biosynthetic process of the cells using carbon intermediates together with reducing power and the energy to make proteins, nucleic acid, polysaccharides and other monomers for making up the new cellular material.

In this review, Ratledge (1991) stated that during catabolism, to balance the supply of carbon with the required reducing power and the energy needed for making new cells, the cell has to oxidise a greater amount of carbon to provide the energy that can subsequently assimilate. Complete degradation of substrate results in the formation of CO₂. The original substrate specifies the amount of CO₂ being released and lost to the cell. If the substrate is high in energy (e.g. carbohydrates), the energy produced from oxidation of the substrate is higher. For substrates such as fats, fatty acids, and hydrocarbons the energy is poor and the carbon content of the substrate is high, energy produced from their oxidation is lower (Linton and Stephenson, 1978). Catabolism of glucose through different metabolic pathways has been well documented (Fraenkel, 1982), Coppella and Dhurjati (1989, 1990).
Biosynthesis, active transport of ionic and neutral substrate through the cell membrane and mobility (work required for the cell division) use chemical energy in the form of ATP and release inorganic phosphate (P_i). The inorganic phosphate is used by the cell to phosphorylate ADP to ATP during the energy yielding reaction of catabolism.

The ATP molecule undergoes very rapid turn over in the ATP-ADP system, in order to transfer energy in the cell.

i) The ADP-ATP system provides the intermediate link between compounds with high and low phosphate group transfer potential, ADP acting as the acceptor and ATP as the donor of the specific enzymatic phosphate group.

ii) The ADP and ATP molecules are used as reactants in all the enzymatic phosphate transfer reactions in the cell, one set of phosphate transferring enzymes catalysing the conversion of the phosphate groups from high potential energy compounds to ADP, and another set of phosphate transferring enzymes catalysing the conversion of phosphate groups from ATP to low energy acceptors.
1.2.2 Glucose metabolism in *S. cerevisiae*

Glucose was the only carbon limiting substrate used in the study demonstrated in this thesis. When metabolised under two different environmental conditions glucose would metabolise to different end products. Glucose metabolism in *S. cerevisiae* has been well documented by many authors including Fiechter and Seghezzi (1992), and Fraenkel (1982).

Glucose molecules transfer into the yeast cell and phosphorylate to produce glucose-6-phosphate. This reaction is catalysed by the hexokinase enzyme. The breakdown of glucose to pyruvate by a series of enzyme catalysed reactions is referred to as Glycolysis. Glycolysis, also known as the Embden-Meyerhof-Parnas (EMP) pathway, illustrated in Figure 1.2, provides energy in the form of ATP, reducing power as NADH or NADPH, and intermediates for biosynthesis of cellular material. The phosphorylated group could also pass down another pathway, called the Pentose-phosphate pathway. The Pentose-phosphate pathway produces the required NADH for biosynthesis of amino acids.

During aerobic respiration, the pyruvate produced in glycolysis is converted to acetyl-CoA and oxaloacetate using the two enzymes pyruvate dehydrogenase and pyruvate carboxylase. The two products from pyruvate combine together to give citrate which is metabolised through a series of reactions known as tri-carboxylic cycle (TCA cycle), to produce carbon dioxide and water (see Figure 1.3). However, under anaerobic conditions, the pyruvate produced could not be metabolised to carbon dioxide and water via the TCA cycle, due to the absence of oxygen, and therefore would be shifted down the fermentation pathway to be reduced to ethanol (see Figure 1.3).

Bellgardt and Yuan, (1991) stated that in the aerobic metabolism, the EMP and TCA pathways can yield up to 36 moles of ATP per mole of glucose, resulting a biomass yield of up to 0.5 g dry cell weight per gram of substrate. Whereas in anaerobic metabolism, the chemical energy would come mainly from glycolysis, with a yield of 2 moles of ATP per mole of substrate. Due to this low efficiency compared to the oxidative growth, and due to the loss of carbon in the product ethanol, the cell yield is also very low. The amount of substrate metabolised to give the same biomass concentration as the aerobic metabolism is therefore much greater in the anaerobic metabolism which is accompanied
by the production of a large amount of carbon dioxide. Therefore, oxidative growth is the preferred metabolic type for *S. cerevisiae* biomass production.

The metabolic energy efficiency is observed in the relative stoichiometric biomass yield on glucose in the presence and absence of oxygen. Sonnleitner and Kappeli (1986) and Barford (1990) have shown that there is a limited respiratory capacity in yeast cells, even when the culture environment is saturated with oxygen. In an oxygen saturated culture, when there is a high glucose concentration, and therefore a high glucose uptake flux into the cell, the limited respiratory capacity of the yeast cells would be exceeded. Overflow of the remaining glucose within the metabolic pathways would occur from respiration to fermentation. This switching of the glucose flux occurs at pyruvate, at the end of the common glycolytic pathway, see Figure 1.3, as the excess glucose is diverted from the fully loaded TCA cycle, to the fermentation pathway to ethanol. Consequently, the yeast cell would be simultaneously operating two parallel pathways for the metabolism of glucose, and ethanol would be produced in the presence of oxygen. This parallel metabolism of glucose is referred to as respiro-fermentative metabolism (see Figure 1.4).

When the glucose uptake flux by the yeast falls, a point would be reached at which the limited respiratory capacity would not be exceeded, the yeast metabolism would then be purely respirative. Variation in glucose metabolism leads to variations in metabolic yields of the free yeast cell suspension in liquid phase culture, even when the culture environment is saturated with oxygen. Hence, the performance of the parallel pathway metabolism has to be taken into account in the assessment of products, since the yeast would not necessarily exhibit a purely respirative behaviour, even when the bioreactor is fully aerobic.

Regulation of the cellular internal glucose concentration in yeast is controlled by both metabolism and glucose transport system. From experimental observations, Barford (1981) indicated the importance of the glucose transport process into and out of the mitochondria. Cortassa and Aon (1994) concluded that sugar uptake has the main role in controlling of the flux ratio of the glycolytic pathway at the branch either towards ethanol production or the TCA cycle at the level of pyruvate. Regulation of glucose transport in *S. cerevisiae* has also been reviewed by Fuhrmann and Volker (1992).

The significance of these mechanisms of glucose metabolism by yeast to this project is the measurement of some intracellular enzymes (ADH, MDH, G6PDH, and hexokinase) that are active during the glucose metabolism as fermentation products.
Figure 1.2 The breakdown of glucose to pyruvate with the simultaneous formation of ATP is referred to as Glycolytic (EM) pathway, adapted from Stryer (1988).
Figure 1.3  Pathway of yeast intermediate metabolism. Pyruvate oxidises by the tri carboxylic cycle (TCA) to carbon dioxide and water. The functions of this pathway are: generation of ATP, generation of reducing power in the form of NADH and formation of intermediates for the biosyntheses of cellular materials.
Glucose, ethanol and oxygen are regarded as substrates. Three defined kinetic structural elements can be distinguished in the cell as:

- The respiratory bottleneck (limited respiratory capacity)
- The total carbohydrate storage material
- The residual biomass (N: nucleic acids, E: enzymes, and St: structural components)
1.2.3 Regulation of glucose metabolism in *S. cerevisiae*

The different pathways of glucose metabolism has been outlined in section 1.2.2. These various pathways have been examined by many workers, in order to try to explain the regulatory mechanisms involved in glucose metabolism. However, the nomenclature used by some researchers to describe these regulatory phenomena are rather confusing. Fiechter *et al.* (1981) classified some common terms used to describe regulation of glucose metabolism in yeast. A number of definitions are as follows (Fiechter *et al*., 1981);

**Growing cells**  Proliferating cells expressed in the form of biomass with simultaneous assimilation of a carbon source present in adequate concentration

**Resting cells**  Non-proliferating cells, no formation of biomass because of incomplete nutritional requirements (mainly starvation of a nitrogen source)

**Repression**  Relative decrease of enzyme formation irrespective of the underlying metabolism

**Derepression**  Relative increase of enzyme formation irrespective of the underlying mechanism

**Pasteur effect**  Inhibition of the glycolytic pathway in the presence of oxygen (manifested as inhibition of ethanol formation)

**Crabtree or Glucose effect**  Repression of respiratory activity by glucose under aerobic conditions and subsequent deregulation of glycolysis with formation of ethanol.

As described in section 1.2.2, glycolysis is the same for fermentative and respirative glucose metabolism. The difference starts at the pyruvate level. In fermentation, decarboxylation of pyruvate, the end product of glycolysis, leads to the formation of acetaldehyde. Alcohol dehydrogenase then catalysis the reduction of acetaldehyde to ethanol. During respiration, pyruvate is oxidatively decarboxylated and coupled to co-enzymeA, thus entering the TCA cycle.
For glucose metabolism in *S. cerevisiae* two regulatory processes are thought to exist, regarding the response of this yeast to increasing concentration of oxygen or increasing concentration of glucose, namely, the Pasteur effect and the Crabtree effect. A full discussion of these effects is given in the review of Gancedo and Serrano (1989).

**Pasteur effect**

Pasteur (1861), reported a decrease in fermentation (ethanol production) efficiency in the presence of oxygen. This behaviour was later referred to as the Pasteur Effect. This effect is only applied to fermentative yeasts (those which produce ethanol as a by-product). Kappeli (1986) reviewed the Pasteur effect in yeast and concluded that there was no satisfactory hypothesis for this phenomena. However, Soles et al., (1971) identified the reaction catalysed by phosphofructokinase as the main regulatory step in the Pasteur effect. This regulation was based on a feedback mechanism, with ATP acting as inhibitor and ADP and AMP being activators of the reaction. However, in their studies of *S. cerevisiae* in a continuous culture, Fiechter and Von Meyenburg (1966) and Von Meyenburg (1969) showed that below a critical dilution rate for pure oxidative glucose turnover, ethanol was not produced at all and only above the critical dilution rate was ethanol detected in the medium. Fiechter and Seghezzi (1992) stated that Pasteur effect does not describe a general metabolic phenomena but rather a single defined state of certain cultures under specialised conditions.

**Crabtree effect**

The Crabtree Effect is used to describe the formation of ethanol under aerobic conditions due to the repression of oxidative metabolism by glucose. This repression would lead to a decrease in the specific rate of oxygen uptake by the cells, and accumulation of ethanol as described by Soumalainen et al., (1973). Fiechter et al. (1981) reported that several enzymes were affected by glucose repression. Ratledge (1991) stated that the degree at which yeast show this effect varies considerably. For some yeasts ethanol accumulates at glucose concentration of as low as 1g/L, for others 20-50 g/L glucose is required to show this effect, and some yeasts don't show this effect at all. Sonnleitner and Kappeli (1986) reported that in yeast cells, production of ethanol in the presence of air is caused by the limited respiratory capacity of the growing yeast cells, when glucose is present in excess, as a consequence of an absent carbon uptake control. Alexander and Jeffries (1990) reported that Crabtree effect appears to be the central regulatory phenomenon for growing of *S. cerevisiae*. 

33
Rieger et al., (1983) proposed a regulatory mechanism, as illustrated in figures 1.5 and 1.6, for glucose uptake in *S. cerevisiae* which seems to be supported by many researchers (Sonnleitner and Kappeli 1986, Alexander and Jeffries 1990, and Fiechter and Seghezzi 1992). The overflow reaction due to the respiratory capacity of the yeast cell, represented as a bottleneck (Sonnleitner and Kappeli, 1986), is used to explain the limited amount of glucose flux that can be oxidised completely. The additional glucose is converted to ethanol and passed into the medium.

Kappeli (1986) and Bradford and Hall (1979) concluded that in *S. cerevisiae* although glucose or catabolite repression accounted for the regulation of certain enzymes, there were many other regulatory mechanisms controlled by other factors such as ethanol, CO₂ and NADH. The complexity of the processes involved in glucose metabolism has therefore lead to misuse of the terms Crabtree and Pasteur effects. Kappeli (1986) suggested that the terms Crabtree effect and Pasteur effects should no longer be applied to *S. cerevisiae*, as these terms were connected with regulatory concepts that did not correspond to the actual situation.

In the review by Fiechter and Seghezzi (1992), these researchers claimed that the confusion caused by the terminology of the Pasteur Effect and presentation of contradictory data in the literature is caused by the fact that no clear differentiation between observations obtained from growing and resting cells was made and, the two cell types were compared directly, and that the Pasteur Effect is only observed in resting cells. Therefore the term Pasteur effect is not recommended to be used to describe general yeast metabolism. Fiechter and Seghezzi (1992) also recommended that the expression 'Crabtree Effect' should not be used to describe the metabolic event of ethanol formation in the presence of oxygen, since in his experiments Crabtree (1929) was working with tumour cells which were probably resting cells when he noticed the formation of lactate in the presence of oxygen.

Repression of the enzymes of the TCA cycle and electron transport chain has been reported for *S. cerevisiae* under conditions of glucose excess (Polakis and Bartly 1965, Beck and von Meyenburg 1968, Fiechter *et al.* 1981). Zeng and Deckwer (1994) concluded that the presence of glucose in the culture represses the activity of the TCA cycle but not the respiratory capacity of *S. cerevisiae*. The authors also concluded that bottlenecks in the oxidative glucose metabolism of *S. cerevisiae* occur at the level of the TCA cycle or beyond.

The effect of the above regulatory phenomenon of glucose metabolism on the aerobic growth of *S. cerevisiae* in batch culture is discussed in section 1.3.
The substrate flux is called subcritical if the total amount of substrate(s) flux can be pass the bottleneck, i.e. substrate is metabolised purely oxidatively and the actual respiratory capacity is not exceeded. The flux is then called critical when the bottleneck is completely filled, the theoretical oxygen demand can just be met by the respiratory capacity. In the supercritical case, when the substrate flux no longer fits into the bottleneck, the following situations must be considered: (1) Glucose flux is supercritical. The substrate flow exceeds the respiratory capacity. The residual part of glucose that can not pass the bottleneck is metabolised reductively (a) and ethanol is excreted into the medium (top right). If additional ethanol is supplied to the medium, the ethanol can not pass the bottleneck because the bottleneck is filled with glucose. Hence the ethanol remains unused (c) because there is no reductive metabolic pathway to utilise ethanol (top right). (2) Glucose flux is subcritical but there is additional ethanol in the medium. Ethanol is utilised oxidatively as long as glucose flux doesn't saturate the cell's respiratory capacity (b), (glucose flux fits (in addition to the fully oxidative flux) into the bottleneck). The residual ethanol can not be metabolised and remains untouched in the medium as soon as saturation occurs (c).
Figure 1.6  Regulation of glucose metabolism in *S.cerevisiae* based on limited respiratory capacity. Glucose flux is degraded oxidatively. When the respiratory capacity is saturated, ethanol formation begins. Activities characteristic of this type of metabolism results. Adapted from Kappeli, (1986).
1.3 Aerobic growth of \textit{S. cerevisiae} in batch culture

In an aerobic batch culture, \textit{Saccharomyces cerevisiae} degrades glucose through a combination of oxidative and reductive metabolisms, referred to as an oxido-reductive metabolism. The choice of the pathway depends on the limitations on the respiratory capacity affected by limitations on oxygen and glucose (Auberson and Stocker, 1992). Oxygen may be limiting either by supply or by biological limitations such as respiratory bottleneck. Reducing the oxygen supply enhances the reductive metabolism, resulting in the formation of increasing proportion of ethanol relative to biomass formation. This shift toward reductive metabolism is a means by which the cell can generate the required ATP through glycolysis, since ATP production from oxidative phosphorylation has been restrained by a decreasing oxygen supply. Glucose may be limiting by respiratory bottleneck which limits the amount of substrate that can be funneled completely through the oxidative pathway (Rieger et al., 1983 and Sonnleitner and Kappeli, 1986).

The diauxic growth pattern of \textit{S. cerevisiae} in an aerobic batch culture has been described by Sonnleitner and Kappeli (1986), Fiechter et al., (1987), and Barford (1990). With glucose as the sole carbon source, the widely accepted view is to divide the batch into two growth phases (see Figure 1.7). During the first growth phase, formation of biomass is associated with the accumulation of ethanol, as glucose metabolises respiro-fermentatively, biomass yield of 0.5 to 0.15 gL\(^{-1}\) dry cell weight (g glucose\(^{-1}\)) was reported. As ethanol is produced, the carbon dioxide production rate is higher than the oxygen uptake rate, leading to a respiratory quotient above unity. After a lag phase of about two hours, during which glucose is totally exhausted, the second growth phase is started with biomass yield of up to 0.35 g.L\(^{-1}\) dry cell weight (g glucose\(^{-1}\)). The respiratory quotient is significantly below unity, since the oxygen uptake rate is higher than the carbon dioxide production rate. RQ approaches the oxidative theoretical value of 0.66. Maximum growth rate was reported to be 0.35 h\(^{-1}\) for the first phase and 0.1 h\(^{-1}\) for the second growth phase.

As discussed in section 1.2.3, the formation of ethanol in aerobic batch cultures of \textit{S. cerevisiae} with glucose as the limiting substrate, was a result of the limited respiratory capacity of the cells which led to an overflow reaction at the pyruvate level when oxidative metabolic pathways were saturated. Beck and Meyenburg (1968) and Sonnleitner and Kappeli (1986) confirmed that this phenomena was due to the rate of glucose uptake rather than the concentration of the glucose in the yeast cell environment. However, the initial
glucose concentration in the aerobic batch culture would influence the uptake flux of glucose due to the existence of different transport systems for glucose through the cell membrane. Hence the occurrence of respiro-fermentative metabolism depends on both the internal metabolic condition of the cells and the external environmental conditions in a batch culture. Environmental variables affecting a fermentation process are discussed in section 1.4.

Figure 1.7-a  Aerobic growth of S. cerevisiae with glucose limiting substrate at an initial concentration of 20 g/L. ▲ indicates biomass (gL⁻¹), ● glucose concentration (gL⁻¹), and ○ ethanol concentration (gL⁻¹). Adapted from Fiechter et al., (1987)

Figure 1.7-b  Oxygen uptake rate ▲ OUR (mmolg⁻¹h⁻¹), carbon dioxide production rate ● CER (mmolg⁻¹h⁻¹), and respiratory quotient ○ of an aerobic batch growth of S. cerevisiae. Adapted from Fiechter et al., (1987).
1.3.1 Repeated batch fermentations

Fill and draw or repeated batch mode of operation is when a proportion of the completed batch is left in the bioreactor to act as an inoculum for the next run. This process operation is usually used in the case of waste water treatment. The method has been effective in large scale production of monoclonal antibodies (Backer et al., 1988), production of industrial enzymes (Salmon and Buckland, 1992), and in recent studies of automatic bioprocess control of S. cerevisiae (Locher et al., 1991, 1993). Repeated batch fermentation is a key component of the work presented in this thesis, and is aimed at providing reproducible fermentations.

In their process for the large scale production of monoclonal antibodies, Backer et al., (1988) reported that the inoculum was initially provided from spinner flasks for the 150L vessel (120L working volume). Every 48h, the bulk of the culture fluid was harvested and approximately 10% was left in the bioreactor to inoculate the next fermentation; the inoculum fraction was adjusted to account for cell concentration and viability. The observation was made that the cell viabilities were generally over 95% through most of the fermentation but may drop about 80% in the last hour before harvest which is the same time as inoculation of the next batch. Backer et al., (1988) suggested an increase in the final antibody concentration could be achieved by extending the fermentation beyond the period of increasing cell concentration; however, this typically resulted in lower cell viabilities and a lag phase upon inoculating the next batch. When the culture was maintained in a constant growth mode, good long term stability of cell growth and antibody characteristics were observed in the system.

In the article on monitoring and control of microbial fermentations, Salmon and Buckland (1992) showed data from a repeated batch fermentation (150 L working volume) for the production of an industrial enzyme, where pH, OUR, and DO were measured and about 30% of the batch were used as inoculum for the next batch. Characteristics of the diauxic growth of the organism was observed in both off-gases and in situ DO measurements. Identification of the transition from these data were used for starting of each new batch. The authors did not state the nature of the microorganism and the enzyme produced, neither did they state the variability of the assay and the profile of the products.
Locher et al., (1991) studied the performance of an automatic bioprocess control of aerobic *S. cerevisiae* in a defined medium containing 3% glucose as a repetitive batch cultivation, where no disturbances or human interactions with the bioprocess were allowed (e.g. no manual sampling of the bioreactor). After each batch 95% of the broth was harvested and the remaining 5% was used as inoculum for the next batch. The bioreactor was refilled automatically using a supervisory computer system that could be triggered either by a timer or by defining a growth phase. The high degree of automation for the repeated batch mode of operation was shown to allow the bioreactor to be used more intensively. The decision making for the refill/fill action was completely automated leaving the equipment unattended most of the time. The repeated batch method was used to investigate the role of different nutrients during growth. Fourteen pulses of 3 different substances in different amounts were applied to a batch culture. The first pulse was triggered by automatic detection of a certain phase whereas the following 13 pulses was configured within 5 min. A high degree of reproducibility was also shown in the partial pressure of ethanol, CO$_2$ exit gas analysis (see Figure 1.8), optical density and redox potential.

In studies of automatic bioprocess control, Locher *et al.*, (1993) used the CO$_2$ exit gas analysis profiles of the repeated batch cultures to subdivid the batch cultivations of *S. cerevisiae* into 7 distinct phases on the basis of metabolic production and consumption (Figure 1.8). Analysis of some extracellular intermediates was also reported. The intracellular intermediates analysis were reported to involve difficult and time consuming extraction procedures, and were not reported in the article.
Phase 1
Cells grow exponentially on glucose with no significant limitations. Ethanol is being produced, excretion of secondary metabolites; glycerol, and organic acids (pyruvic acid, acetic acid, and propionic acid) are also reported.

Phase 2
The CO₂ measured in the exhaust gas is no longer exponential. This effect was not observed when yeast extract was added or the bioreactor was stirred less vigorously.

Phase 3
Glucose is exhausted and cells start utilising pyruvic acid.

Phase 4
Utilisation of acetic acid, glycerol, and to a minor extent propionic acid is the characteristic of this phase.

Phase 5
Phases 5 and 6 are ethanol dominated. Acetic acid is excreted and accumulates until ethanol is completely exhausted. Glycerol is also reutilised.

Phase 6
This phase is mainly caused by ethanol (and glycerol) consumption in combination with acetic acid excretion.

Phase 7
This phase is determined by the deplition of ethanol and glycerol and the subsequent utilisation of acetic acid as the final carbon source which is left in the medium.
1.3.4 Fermentation products

When grown under aerobic conditions with glucose as the sole energy and carbon sources, \textit{S.cerevisiae} produces the necessary enzymes to metabolise glucose and releases ethanol as a by product. Although biomass is usually the main product of Baker's yeast fermentations and ethanol is considered to be the main by product of glycolysis in \textit{S. cerevisiae}, a considerable range of enzymes and other intracellular components are also produced by yeast (Peppler, 1977).

Yeast enzymes may generally be divided into two categories of commercial importance (Barford, 1987). Enzymes with significant importance to the food industry and others which are used as chemicals in laboratories. Invertase, lactase and some production of lipase are the major enzymes in the first category. Enzymes which may be categorised as fine chemicals are alcohol dehydrogenase, aldehyde dehydrogenase, glucose-6 phosphate dehydrogenase, alpha-glucosidase and hexokinase. The use of these enzymes are listed as assay of ethanol for ADH, generation of NADP+ by G6PDH, and assay of malate for MDH enzyme (Atkinson and Mavituna, 1991).

Formation of glucose-6-phosphate dehydrogenase (G6PDH), hexokinase, alcohol dehydrogenase (ADH) and malate dehydrogenase (MDH) intracellular enzymes (see Figure 1.3) were considered as products of fermentations to seek correlations between these metabolic indicators and monitored variables for on-line recognition of fermentation phase transition decision making and optimisation of the process.

In the investigation of the enzyme patterns in an aerobic batch culture of \textit{S.cerevisiae} grown on glucose, Beck and Meyenburg (1968), reported activity profiles of seven enzymes. During the first growth phase with a cellular specific growth rate of 0.42 h\(^{-1}\), succinate-cytochrome c oxidoreductase, malate dehydrogenase, NAD-linked glutamate dehydrogenase, malate synthase, isocitrate lyase were reported to be repressed, whereas aldose and NADP\(^+\)-linked glutamate dehydrogenase were shown to be derepressed. During the second growth phase with a cellular specific growth rate of 0.14 h\(^{-1}\) the first five enzymes were formed and the other two were repressed. These researchers also stated that, although the activity of aldose, the enzyme of glycolysis, was reduced by about two thirds in the second exponential growth phase, the enzymes responsible for the TCA
cycle were increased by a factor of 10 in the case of succinate-cytochrome c and by a factor of 20 in the case of MDH.

Polakis and Bartley, (1965) studied the changes in enzyme activities of *S. cerevisiae* during aerobic growth on different carbon sources. Table 1.1 shows the enzyme activity of the cells grown in a synthetic medium with glucose as the carbon source. At all stages, malate dehydrogenase was reported to be the most active enzyme of the TCA cycle. For the cells grown on glucose in a synthetic medium, during the first exponential stage of growth the cells had the lowest MDH activity and in the second exponential stage of growth an increase in the activity of about 6-7 fold was observed in the cells for this enzyme.

<table>
<thead>
<tr>
<th>Time of growth (h)</th>
<th>First stage exponential growth</th>
<th>Second stage exponential growth</th>
<th>Late Second exponential growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>123</td>
<td>197</td>
<td>128</td>
</tr>
<tr>
<td>15</td>
<td>6580</td>
<td>2520</td>
<td>2280</td>
</tr>
<tr>
<td>30</td>
<td>3.8</td>
<td>7.5</td>
<td>6.8</td>
</tr>
<tr>
<td>First stage</td>
<td>17.5</td>
<td>20.8</td>
<td>26.1</td>
</tr>
<tr>
<td>Second stage</td>
<td>17.6</td>
<td>33.4</td>
<td>30.8</td>
</tr>
<tr>
<td>Growth phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>25.9</td>
<td>72.3</td>
<td>69.8</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme (μmoles/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>6.8</td>
<td>7.8</td>
<td>9.1</td>
</tr>
<tr>
<td>Pyruvate decarboxylase</td>
<td>211</td>
<td>407</td>
<td>423</td>
</tr>
<tr>
<td>Pyruvate-oxidase system</td>
<td>450</td>
<td>2860</td>
<td>3060</td>
</tr>
<tr>
<td>Acetyle-CoA kinase</td>
<td>3.8</td>
<td>41.4</td>
<td>56.2</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase (NAD+ linked)</td>
<td>17.5</td>
<td>7.5</td>
<td>6.8</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase (NADP+ linked)</td>
<td>17.6</td>
<td>33.4</td>
<td>30.8</td>
</tr>
<tr>
<td>Succinyl-CoA synthetase (ATP)</td>
<td>25.9</td>
<td>72.3</td>
<td>69.8</td>
</tr>
<tr>
<td>Fumarase</td>
<td>6.8</td>
<td>7.8</td>
<td>9.1</td>
</tr>
<tr>
<td>211</td>
<td>407</td>
<td>423</td>
<td></td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>450</td>
<td>2860</td>
<td>3060</td>
</tr>
<tr>
<td>Isocitrate lyase</td>
<td>&lt;1</td>
<td>41.4</td>
<td>56.2</td>
</tr>
<tr>
<td>Malate synthase</td>
<td>&lt;1</td>
<td>16.9</td>
<td>22.3</td>
</tr>
<tr>
<td>Glutamate dehydrogenase (NAD+ linked)</td>
<td>1</td>
<td>30.6</td>
<td>26.5</td>
</tr>
<tr>
<td>2910</td>
<td>1600</td>
<td>1630</td>
<td></td>
</tr>
<tr>
<td>Glutamate dehydrogenase (NADP+ linked)</td>
<td>148</td>
<td>126</td>
<td>116</td>
</tr>
<tr>
<td>Glutamate-oxaloacetate transaminase</td>
<td>3.8</td>
<td>26</td>
<td>20.3</td>
</tr>
</tbody>
</table>

Table 1.1 Changes in the activity of enzymes during growth of yeast (Adapted from Polakis and Bartley, 1965).
1.3.5 Inhibition by fermentation products

Ethanol, the main product of glycolysis in *S.cerevisiae*, is known to inhibit fermentations, to cause unfavourable effects in yeast cells such as decreasing the growth rate and reducing cell viability (Aiba *et al.*, 1968). Apparent toxicity of ethanol on the performance of *S.cerevisiae* (van Uden, 1985) is higher when ethanol is produced during aerobic fermentation than when ethanol is added to the medium (Nagodawithana and Steinkraus, 1976). Loureiro and Ferreira (1983) demonstrated that intracellular accumulation of ethanol does not occur. When ethanol was used as substrate, substrate inhibition was found to occur when the ethanol concentration was above 150 g/L. Organic acids, aldehydes, higher alcohols, and fatty acids produced by yeast may accumulate during fermentation (Maiorella *et al.* 1983, Lafon-Lafourcade *et al.* 1984) and act as inhibitors. Locher *et al.* (1993) indicated that acetic acid and propionic acid must not exceed a certain concentrations (of the order of 3 g/L), otherwise growth of *S.cerevisiae* is inhibited. These workers also reported that the inhibition could not be observed for pyruvic acid in concentrations up to 4 g/L. Pampulha and Loureiro (1989) observed higher toxicity of acetic acid in the presence of ethanol and stated that yeast fermentations may be significantly affected by acetic acid, principally during the last stages of the process since the concentration of ethanol was already high.

Phosphofructokinase inhibits hexokinase activity (Stryer, 1988). Phosphofructokinase, the enzyme catalysing the phosphorylation of fructose-6-phosphate to fructose-1,6-biphosphate (the first irreversible reaction in the glycolytic pathway) can be blocked by high levels of ATP, H+, citrate, and early intermediates of the citric acid cycle. The level of fructose-6-phosphate increases when phosphofructokinase is blocked. In turn, the level of glucose-6-phosphate rises because this phosphate is in equilibrium with fructose-6-phosphate. Accumulation of glucose-6-phosphate has been reported to suppress the glucose uptake mechanism (Sols *et al.*, 1971). However glucose-6-phosphate can be oxidised by the pentose phosphate pathway to generate NADPH.

Pascaul *et al.*, (1988) examined the effect of ethanol on the activities of key enzymes of the glycolytic pathway and on the glucose uptake system with fermentations of *S. cerevisiae*. A significant decrease in CO2 liberation was observed in the presence of 1M ethanol. In the presence of 2M ethanol, although the fermentation rate was reduced by 50%, the glucose uptake rate was reported to have stayed the same. Therefore inhibition by a higher ethanol concentrations could be attributed to an alterations in the plasma membrane in the
presence of ethanol. Growth stopped with 3M ethanol. The effect of ethanol on the activities of the glycolytic pathway enzymes, in cell extract and whole cells, showed the influence of higher ethanol concentration for inhibition of these key enzymes in whole cells than that of cell extracts (see Table 1.2). Ethanol was incubated with cell extracts and analysed for enzyme activity levels. With 3M ethanol considerable inactivation of pyruvate kinase and to a lesser degree of hexokinase was reported. G6PDH was also effected. At 4M inactivation of all enzymes except phosphofructokinase was reported. When whole cells were incubated with ethanol a pattern of enzyme inactivation at 5M ethanol was reported similar to that described for cell extract at 3M ethanol. Therefore phosphofructokinase was least affected, while pyruvate kinase was most affected followed by hexokinase.

<table>
<thead>
<tr>
<th>Ethanol concentration</th>
<th>0M</th>
<th>1M</th>
<th>2M</th>
<th>3M</th>
<th>4M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition to cell extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexokinase</td>
<td>0.34</td>
<td>0.24</td>
<td>0.29</td>
<td>0.12</td>
<td>0.00</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>0.36</td>
<td>0.43</td>
<td>0.30</td>
<td>0.33</td>
<td>0.57</td>
</tr>
<tr>
<td>Aldolase</td>
<td>0.64</td>
<td>0.77</td>
<td>0.73</td>
<td>0.52</td>
<td>0.00</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>3.55</td>
<td>3.77</td>
<td>2.24</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>0.09</td>
<td>0.11</td>
<td>0.12</td>
<td>0.05</td>
<td>0.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>incubation of whole cells with ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
</tr>
<tr>
<td>Aldolase</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
</tbody>
</table>

Table 1.2 Effect of ethanol on specific enzyme activities of yeast *S.cerevisiae* in cell extract and whole cells (Adapted from Pascual et al., 1988). Specific enzyme activities are expressed in μmol/min/mg protein.
1.4 Fermentation environmental variables

Environmental variables can influence the growth of living cells which affects the optimisation of biological processes. Accurate monitoring and control of these variables is reported (Carleysmith and Fox 1984 and Carlysmith 1988). Fiechter (1984) reviewed the possible effects of the environment on the behaviour of the microbial cell during growth in submerged culture. The effectors of microbial growth have been categorised into those that are intracellular and those that are extracellular. The intracellular variables consist of the genome and the apparatus for replication, transcription and translation. The genome is taken as a constant as long as the strain used is not replaced by any other type of cell. The extracellular parameters can be divided into the two categories of physical and chemical measurements.

1.4.1 Physical and chemical variables

Montague et al. (1989) described physical measurements as volume of the bioreactor, temperature, pH, pressure, agitation, viscosity, and chemical measurements such as volumetric gas flow rates, liquid flow rates (acid, base, antifoam and nutrient feeds ), dissolved oxygen transfer, nutrient concentration, redox potential, product and biomass concentration, and rheological measurements. Some fermentation variables can be measured on a continuous basis with some degree of reliability and are used to maintain the desirable environmental conditions of the process. Conditions an be maintained reasonably close to the the desired values (e.g. ±0.1°C, ±0.1 pH ). However, the major problem is knowing the most desirable operating condition. In order to optimise the performance of the process, operating conditions are often chosen based on past experience and experimentation approaches.

Microorganisms respond differently to fermentation variables. The optimum measured value of these variables for growth rate may be different from that for growth yield and entirely different from the optimum value for product formation. For penicillin production a higher penicillin yield is obtained by starting at a temperature of 30°C, followed by operating at a constant, low, temperature of 20°C to 25°C compared to a controlled fermentation at 25°C (McCann and Calam, 1972). Andreyeva et al., (1973) discussed the effect of pH on growth and penicillin production and stated that the optimum pH profile should not be constant as a rule, since the optimum value is likely to be different for growth and product formation. Different pH values for a maximum specific oxygen
uptake rate for 66 hours (pH=6.7) and 90 hours (pH=7.0) was observed with growth on synthetic media.

The difficulty arises in that changing one variable would lead to a change in a number of other variables. A change in pH can affect the evolution of CO$_2$ which affects the mass balances and biomass estimations (Royce, 1993). Montague et al., (1989) demonstrated the major interaction that can take place between variables within a fermentation (Figure 1.9), and stated that other interdependences could also be postulated. Many of these interactions have been modelled mathematically.

![Figure 1.9 Typical Bioprocess Interactions (Montague et al., 1989).](image)

The reproducibility of bioprocesses is mainly dependent on the reproducibility of environmental conditions for the cells, and therefore on the quality of the equipment. In a batch culture, a strict control of the physical parameters like pH, temperature, pressure, or aeration is not sufficient to obtain reproducible results due to the continuous change in biomass, substrate, and product concentrations. Control over the medium composition, inoculum (state, size, and volume), and other environmental variables are also important. A good reproducibility of fermentation results has been reported from repetitive aerobic batch fermentations of yeast *S.cerevisiae* by Locher et al. (1991).

Effect of environmental variables on growth and optimisation of yeast *S.cerevisiae* has been extensively studied by many authors. Yeast grows quite well at temperatures between 28°C to 32°C and at pH levels between 3.6 and 6.5 (Chen and Chiger, 1985). Changes in environmental variables such as oxygen or substrate concentration may alter the yeast's metabolism from oxidative to oxido-reductive (Auberson and Stocker, 1992).
Nutrient concentration (Kappeli, 1986) can also affect the metabolism. Kalle and Naik (1987) studied the effect of controlled aeration on glycerol production in *S. cerevisiae*. Increasing aeration (1-1.4 vvm), improved the ability of the strain to metabolise higher concentrations of sugar in the medium (400-465 g/L), which led to a better growth rate and higher productivity of glycerol by threefold. While at aeration rates greater than 1.4 vvm productivity was reported to decline due to the general inhibition of the fermentation.

Ahmad *et al.*, (1991) studied the effect of some environmental variables on growth characteristics of *Candida utilis*. The lag phase of the growth curve was reported to increase with initial sugar concentration, and to decrease with an increase in inoculum dosage and agitation speed. Increasing inoculum dosage, air flow and agitation speed was reported to increase the overall yield (0.27 at 200 rpm and 0.57 at 700 rpm; 0.28 at 21 L/min and 0.45 at 1.2 L/min).

Abel *et al.* (1994) investigated the effects of variations in dissolved oxygen concentration in the micro environment of yeast cells on their physiological behaviour. The gas composition was changed periodically by varying the flowrate ratios of the air to nitrogen gas, keeping the flowrate of the gas mixture constant. For the batch cultivation on glucose the results showed that, cell growth rate on glucose, and the final cell concentrations were higher for the experiment with a higher aeration frequency (0.79 min⁻¹) and dissolved oxygen concentration between 30 and 2% than experiments with an aeration frequency of 0.56 min⁻¹ and dissolved oxygen concentration between 55 and 5%. The conclusion was made that the influence of periodical changes of dissolved oxygen concentration decreased with increasing frequency, due to the slow response of the cells to variations in the environment. Abel *et al.* (1994) indicated that as a consequence of the periodically changing gas composition and dissolved oxygen concentration, aerobic and anaerobic phases interchange. During the aerobic phase the respiratory capacity is high and glucose is metabolised oxidatively due to a subcritical glucose flux. The residual respiratory capacity is used for consumption of ethanol. The researchers concluded that at a high frequency of the gas consumption change, the respiratory capacity of the cells might not be able to respond to the dissolved oxygen concentration changes, thus, an intermediate respiratory capacity is followed according to the average dissolved oxygen concentration. At low frequencies of dissolved oxygen variation, however, the respiratory capacity changes periodically causing the growth rate on ethanol and the yield coefficient of the growth on ethanol as well as cell concentration to decrease.

The applied environmental conditions have a direct impact on the properties of the yeast as a product of fermentation. For example higher temperatures tend to give drier yeast, and at lower pH value a cream coloured instead of a white yeast (Burrows, 1970) is produced.
1.4.2 Measurement of fermentation variables

Although the computer capability has helped controlling bioreactors, lack of reliable sensors has limited the use of computers for feed back control. In order to control biochemical processes there is a need to determine the current state of the process. In the case of fermentation processes the actual state includes components which are difficult and time consuming to measure on-line. Schugerl (1991) reviewed the common instruments for process analysis and control. Armiger (1985) categorised fermentation measurements into in-line, on-line, and off-line measurements. Off-line measurements were defined as systems that involve removal of a sample at periodic intervals for subsequent treatments and analysis. In-line measurements were described as those made by direct reading probes in a non-destructive manner within the physical environment of the bioreactor (e.g., temperature, pressure, liquid and gas flowrates, pH, DOT). The third category, on-line measurement, was outlined as measurements made by sensors that are not in direct contact with the process (such as off-gas analysis and measurement of volatile components). Bull (1985) divided fermentation variables into three categories of; principle variables which can be measured on-line in a bioreactor (temperature, agitation, exhaust gas, etc.), more important quantities which can be measure off-line (sugars and other substrate concentrations, enzyme activities, biomass, etc.), and variables which can be calculated in a relatively straightforward way using the results of on-line and off-line measurements (OUR, CER, biomass, products, by-products, etc.).

There is still the need for reliable on-line sensing devices which can, continuously and rapidly, measure variables such as concentration of chemical components inside the fermentation broth. This requirement has led to the necessity of improvement of on-line measurements. On-line monitoring of fermentation variables other than the routine on-line measurements of DOT, pH, temperature has proved difficult. Although more methods of measuring the state of fermentations on-line are becoming available. Instruments for automatic sampling of bioreactors (Bradley et al. 1991, Oakley 1990, Strudsholm et al. 1992), sterilisable sensors such as the glucose probe (Brooks et al. 1988) and biomass probes (Harris et al., 1987) are now available. On-line viscometry for mycelia measurements (Kemblowski et al. 1985, Endo et al. 1990) and on-line determination of intracellular enzyme activities using flow injection analysis (Kracke-Helm et al. 1991, Steube and Spohn 1994) have been described.

Techniques such as high performance liquid chromatography (HPLC) has also shown some success in measuring the chemical compounds of the broth. The method requires automatic sampling and separation techniques for the broth to separate the solid and liquid components to produce a clarified sample for the HPLC column. Therefore the method is time consuming and expensive. Dincer et al., (1983) reported the HPLC to be a viable
compositional sensor for fermentation processes. Turner et al. (1993), developed an online monitoring system to automate measurements of sugars and organic acids and to demonstrate the effectiveness of the system in controlling a fermentation process. On-line measurement of protein production has also been described for enzyme analysis such as β-galactosidase, a cytoplasmic enzyme frequently monitored in E.coli fermentation broth (Valero et al., 1992, Kracke-Helm et al., 1991).

On-line mass balancing from the bioreactor exhaust gas analysis can give an indirect indication of the state of the process. Mass spectrometry and other techniques such as paramagnetic analysers for O₂ and CO₂ and gas chromatography are available for on-line monitoring of fermentation exhaust gas. Mass spectrometry has been proved to be able to determine the consumption and production rates of gases and volatile compounds of fermentation processes. The analysis method has also provided a means for early detection of microbial contamination at the inoculum stage. Advantages of this technique are: rapid response time (<1 min.), high sensitivity, the ability to analyse several components simultaneously, linear response over a broad concentration range, minimal calibration drift, possibility of connecting to several fermentations, and ease of storing results in a computer. In addition to the analysis of exit gas various volatile components such as ethanol, methanol, acetate, volatile fatty acids and ammonia may be monitored (Kuhlman et al. 1984, Coppella and Dhurjati 1987, Reuss 1988, Camelbeeck et al. 1988, Heinzle 1987 and 1992). Computerisation of industrial fermentation plants together with the mass spectrophotometer has made possible the detailed on-line analysis of process data to obtain meaningful information on the kinetics and physiology of the fermentation. Buckland (1985) stated that the results obtained from mass spectrometry have proved invaluable, and these data provide primary indication of microbial activity in any fermentation. On-line measurements of fermentation exhaust gas and volatile compounds from a mass spectrometer can be sent directly to a computer where it would be possible to generate valuable derived data (Buckland, 1985) such as oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and respiratory quotient (RQ) with a high degree of accuracy by setting up appropriate elemental balances without causing contamination (Chen et al. 1986).

Many of the compounds that are important for fermentation monitoring and control, including substrates, biomass, and enzymes, can not be measured on-line and are absent from the off-gas analysis. Analysis of these compounds requires off-line sampling of the broth. Off-line measurements are time consuming and normally require a great deal of man power. Recently, robotic systems have been developed and used for the analysis of whole broth (Reda et al. 1991). Salmon and Buckland (1992) stated that the robots do not require detailed programming and simple commands are used to define the sequence of operations.
1.4.3 Accuracy and reliability of measurement variables

Accuracy and precision of measured data is important for use in process control. Accuracy of the measurements is expressed as the difference between the observed value of the variable and its true value and precision related to the probability that repeated measurement of the same system will produce the same values. The distribution of the values around their mean is usually characterised by the variance or standard deviation (Schugerl, 1991). Flynn (1982) reported results on the performance of the instruments in a 1m³ pilot plant bioreactor (Table 1.3). Schugerl, (1991) commented on the reported air flow control to have low accuracy and stated that a much higher accuracy and precision should be obtained for this measurement, provided the right instrument is used (e.g. mass flow meter). The accuracy of the dissolved oxygen partial pressure (pO₂) was also reported to be very low. Better accuracy and precision for pO₂ measurements was suggested by using three sensors and parameter-adaptive control (Schugerl, 1991).

<table>
<thead>
<tr>
<th></th>
<th>Accuracy</th>
<th>Precision (stand. dev.)</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measurement</td>
<td>±0.1 W</td>
<td>0.02</td>
<td>0.02 °C</td>
</tr>
<tr>
<td>Control</td>
<td>0.02</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measurement</td>
<td>±0.1 4H</td>
<td>0.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Control</td>
<td>0.1</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td><strong>pO₂</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measurement</td>
<td>6.0 R</td>
<td>0.52</td>
<td>0.05 mbar</td>
</tr>
<tr>
<td>Control</td>
<td>0.52</td>
<td>9.95</td>
<td></td>
</tr>
<tr>
<td><strong>Pressure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measurement</td>
<td>0.41 R</td>
<td>0.07</td>
<td>0.004 psig</td>
</tr>
<tr>
<td>Control</td>
<td>0.07</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td><strong>Volume</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measurement</td>
<td>0.4 R</td>
<td>3.4</td>
<td>0.14 L</td>
</tr>
<tr>
<td>Control</td>
<td>0.4</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td><strong>Air flow</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measurement</td>
<td>-7.0 R</td>
<td>23.0</td>
<td>0.02 L/M</td>
</tr>
<tr>
<td>Control</td>
<td>-23.0</td>
<td>23.0</td>
<td></td>
</tr>
<tr>
<td><strong>Exit CO₂</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measurement</td>
<td>±0.1 R</td>
<td>0.0005%</td>
<td></td>
</tr>
<tr>
<td><strong>Exit O₂</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measurement</td>
<td>±0.1 R</td>
<td>0.0005%</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.3 Performance of measurement and control instrumentation in a 1 m³ pilot plant bioreactor (Flynn, 1982). W, weekly; H hourly; R per run, which relates to the frequency with which the measuring instruments are calibrated.
Heinzle and Dunn, (1991) described two types of error for gas analysis:

a) *Measurement error*

This type of error depends on the equipment used and is identified as:

i) calibration offset errors that can be minimised by using accurate standards and by careful calibration procedures.

ii) instrument drift can be minimised by application of high quality instruments like mass spectrometry, and measurement noise which may be identified and compensated by application of estimators with dynamic filters (e.g. Kalman filter) including a measurement model or artificial intelligence system.

b) *Errors caused by simplification of gas balancing*

This type of error can be avoided by proper setting up of balance equations and by careful analysis of the system.

Heinzle *et al.*, (1990) stated that when gas analysis is used indirectly in combination with other measurements to estimate other concentration values and growth and production rates, measurement errors may amplify dramatically and lead to misleading results. For example a 1% relative offset calibration error for oxygen was reported to result in an error of >50% for poly-β-hydroxybutyrate (PHB) production by *Alcaligenes latus* fermentations. Heinzle *et al.*, (1990) suggested that error propagation for the fermentation was caused by the fact that the degree of reductance in substrate, biomass and PHB was not sufficiently different whereas in cases like production of ethanol using Baker's yeast a much larger difference in the degree of reduction exists. The following precision values (vol. %) based on a quadrupole mass spectrometer during cultivation of *Bacillus subtilis* was also reported: nitrogen (0.024), oxygen (0.020), argon (0.0011), and carbon dioxide (0.0034). These values, combined with automatic recalibration, was reported to be sufficient for reasonable estimation of PHB, biomass and substrate.

Buckland, (1985) reported an accuracy of ± 0.1% and calibration drift of less than 1% per month for a mass spectrometer with a magnetic sector of fixed field strength. Therefore mass spectrometry is highly accurate and reliable.
1.5 Fermentation measurements for control

Zabriskie, (1985) divided fermentation variables into three categories of direct measurements, indirect measurements, and estimated variables. The term direct measurement was used to describe data obtained from sensors, indirect measurements were described as combinations of measured variables defined by a formula common to all fermentations which are specially useful for data interpretation (CER and OUR are examples of indirect variables), and estimated variables were referred to as quantities which can not be determined directly using a sensor or employing a simple formula (such as biomass concentration). Fermentation models (Thornhill and Royce, 1991) and computer power (Buckland, 1990) has helped the application of these variables in control of fermentation processes. Omstead et al., (1990) outlined the range of computer applications of direct and derived measurements in fermentation control are described in this section (Table 1.4).

<table>
<thead>
<tr>
<th>Monitoring and control of direct measured sensor data</th>
<th>Quantitation and control of indirect measured parameters</th>
<th>Quantitation and control of liquid phase composition</th>
<th>Integrated parameter control</th>
<th>Control of process events</th>
<th>Information archival</th>
</tr>
</thead>
<tbody>
<tr>
<td>(temperature, pressure, agitation, aeration, pH)</td>
<td>(mass transfer coefficient, respiratory quotient, biomass)</td>
<td>(substrate and product composition, volumetric use rates)</td>
<td>(specific uptake and use rates, carbon and energy balances)</td>
<td>(inoculum transfer, sterilisation)</td>
<td>(on-line collected data, off-line data)</td>
</tr>
</tbody>
</table>

Table 1.4 Integration of computer capabilities (Adapted from Omstead, 1991)
1.5.1 Direct fermentation variables

Direct measurements of temperature and pH have been used to control fermentation processes. Cheruy and Durand (1979) investigated maximisation of erythromycin biosynthesis in a batch culture by evaluating optimal temperature and pH profiles. Robbins and Taylor (1989) used pH as the monitored variable during *E. coli* fermentation for optimal growth by manipulating glucose feed rate. Andreyeva *et al.*, (1973) used an optimum pH profile to maximise batch production of penicillin G. Pan *et al.* (1972) reported the control of carbohydrate and nitrogen feed rates by pH measurement for production of penicillin G. The nitrogen source was metabolised to basic cations and the carbohydrate source to CO$_2$ and organic acids. The balance of the two ingredients provided the basis for pH control. Stephanopoulos and San (1984) also proposed a relationship between the total rate of biomass growth and ammonium addition to the reactor for pH control.

Information available from direct process measurements is used to develop expert systems.

1.5.1.1 Data bases and neural networks

Data base and neural network applications have been used for analysing fermentation data to control bioprocesses. Data bases have been used for storing fermentation data from a number of experimental batches. Comparative reasoning tools such as linearisation of data can then be used to interpret the data (Marshall *et al.* 1992). Neural networks require fermentation data from previous runs for training the neural network to recognise patterns in the data. The trained neural network is then used on-line to interpret data from a new fermentation. Although the method is being investigated by many researchers (eg. Raju and Cooney, 1992; Jalel *et al.*, 1992; Chen, 1992; Di Massimo-Peel *et al.*, 1992; Ruenglertpanyakul *et al.*, 1992 ), there is yet the question of whether neural network is the best answer to the fermentation data analysis problems.
1.5.2 Derived fermentation variables

Several authors have considered the application of derived or indirect measurements of carbon dioxide evolution rate (CER) and oxygen uptake rate (OUR) for on-line estimation of cell growth.

Calculations of oxygen uptake rate have been used to estimate the cell concentration of *Streptomyces avermitilis* during the active growth phase of the fermentation (Gbewonyo *et al.*, 1989). During the time course of a fermentation, a large amount of data from the process is generated and stored. Gbewonyo *et al.* (1989) demonstrated a remarkably good correlation between dried cell weight and OUR during the growth phase of *Streptomyces avermitilis*, which indicates an example in which OUR can be used to predict biomass provided that during the growth phase all oxygen consumed is directed into cell mass formation.

Wang *et al.* (1977) used gas flow rate and ammonium addition rate of a Baker's yeast fermentation to calculate CER and OUR for on-line mass balancing to estimate the biomass concentration. Mou and Cooney, (1983) used similar data to develop a computer aided methodology for on-line monitoring and control of cell growth in fed batch penicillin fermentations. Zhong *et al.* (1994) established a computer aided on-line real-time monitoring system for plant cell bioprocesses. The system calculated variables such as RQ, CER, and OUR for the identification of the physiological states of the cells during cultivation. The researchers also reported that in their system OUR may depend on the accumulation of a secondary metabolite.

Park *et al.* (1983) found a linear relationship between CER and cell concentration of *Brevibacterium divaricatum* for production of glutamic acid which made it possible to determine specific growth rate and specific CER by on-line measurements of CER with time. Wu *et al.* (1988) stated that the linear proportionality is only valid for cells growth during the exponential growth phase. Wu *et al.* (1988) developed an on-line estimation of the cell mass based on the balances of materials for glutamic acid production. Cell mass was calculated with the assumption that cells do not grow any more when the glutamic acid is produced. A maximum deviation of the estimated data from the measured data was reported to be about 3%. Therefore their method is a satisfactory one for glutamic acid fermentations. However this simple relationship can not be developed for most fermentation processes (Omstead *et al.*, 1990). In the case of yeast fermentations RQ value fluctuates during batch growth on glucose. A dramatic decrease in RQ occurs without significant changes in biomass as glucose is exhausted and ethanol becomes the...
principle carbon source, a direct correlation of biomass with CER and OUR can not be made. Wang et al., (1977) developed a microscopic balancing method for cell mass prediction. The method is applicable in fermentations where biomass accumulated is not directly related to CER or OUR.

RQ is a useful guide to the metabolic state of a yeast fermentation (Wang et al., 1977; Berry and Brown, 1987), and thus a valuable secondary control parameter when it is essential to minimise ethanol concentration, as in Baker's yeast production, or to maximise ethanol production, as in brewing. RQ was reported to be greater than unity when ethanol is being produced in a yeast fermentation as a result of high glucose concentration and/or low oxygen concentration. With aerobic growth on low levels of glucose, insufficient to cause glucose repression, RQ is nominally unity. Fieschko et al., (1987) developed a fed batch protocol to grow *S.cerevisiae* containing recombinant plasmid to dry cell weight densities of up to 100 g/L. The yeast cells were grown batchwise (in minimal medium) to a cell concentration of 0.6 to 1.1 g dry cell per litre at which point the addition of nutrient feed was started. During this operation, the concentration of both glucose and ethanol were always less than 100 mg per litre and RQ remained between 1.0 and 1.2. Rate of nutrient addition had to be restricted and cell densities were kept below 100 g/L to avoid oxygen transfer problems.

1.5.2.1 Fermentation phase profiling

Several authors have reported detailed mathematical models which apply well during the microbial growth of the process, while others have recognised that different phases may need different models which have then to be linked together.

Phase recognition profiling, can be used as a fermentation runs through the distinct, identifiable phases (Winkler, 1991). During the time course of a batch fermentation there are phases associated with the formation of biomass and production phase. There are also other phases associated with the formation of precursors or intermediate products where biomass itself is not the required product. Different phases can also be subdivided into distinct stages. Biomass growth phase can be subdivided into different stages, with changes in specific growth rate, actual growth rate, or respiration quotient, for example, indicating changes in metabolic activity.
An example of a successful application of phase recognition profiling was reported (Lundell, 1982) for the optimisation of extracellular enzyme β-galactosidase in batch culture of Aspergillus niger. Based on CER and RQ, the pH and temperature settings were changed from those of optimal biomass growth (36°C, pH4.8) to those for β-galactosidase production (35°C, pH4.5). Slowing of biomass growth was indicated by a 20% fall in RQ and CER in which time the additional carbon source was fed to the fermentation to extend the growth phase and maximise the biomass concentration. The rapid fall in CER and RQ indicated the onset of the enzyme formation phase. The computer control system then changed the values of pH and temperature. Rate of formation of β-galactose was analysed on line, and when a significant decrease was observed, an inducer was added. Fermentation was terminated when the maximum level of enzyme was attained. The result was a threefold increase in the enzyme yield, a doubling time of the process productivity, and a 50% decrease in energy consumption compared with the conventional batch fermentation.

Horiuchi et al. (1992) reported the development of an on-line control system of a bioreactor with culture phase recognition based on a fuzzy set theory for a fed batch α-amylase production. In this system, the time course of the fed batch culture was classified into five phases of lag phase, transition phase, amylase production phase (I), amylase production phase (II), and sporulation phase. The state variables of the bioreactor were culture time, optical density as cell concentration, and CO₂ content of the exhaust gas which were used to identify culture phase. The control variable was the glucose solution feeding rate. The system was proved appropriately recognise culture phases during the fed batch culture to give a suitable control policy for the substrate feeding rate which was modified every five minutes.

A method to control transition between functional phases applied to alpha-amylase and yeast fermentations has been proposed (Halme and Visala, 1991) using a "wire" model. Functional phases are the different fermentation phases such as lag phase, growth phase, stationary phase, or in the case of B. subtilis for production of alpha amylase these are lag phase, growth phase (I), intermediate phase, growth phase(II) and the declining phase (Halme, 1988). The number of functional phases are finite and usually low. When the functional state changes a structured change happens and the behaviour of the system changes from one phase to another through various permitted transitions. The permitted transitions depend on the history preceding the present state as well as on various logical conditions applying at the time of the transition. The permitted paths between phases are illustrated as wires joining the permitted phases. In this thesis, profiles from the CO₂ content of the exhaust gas are used to identify transition of culture phases and product schedules. The on-line process decision making system is based on the correlations of intracellular enzyme products with CO₂ exit gas analysis.
1.6 Summary

The literature review presented in this chapter illustrates the complexity of even the simple and extensively investigated aerobic yeast batch culture system. The complexity exists not only in terms of interaction between the yeast cell and the culture environment but also with reference to the flux rate of both sugars, as glucose, and dissolved oxygen from that environment into the cell. The supply of glucose and oxygen initially and primarily influences energy generation by yeast cell that results in the production of the ATP molecule. The relative supply of oxygen and glucose to the yeast cells, and within the yeast cells to the mitochondria dictate, which energy yielding metabolism is active within the cell, that is respiration from pyruvate to the TCA cycle, or fermentation from pyruvate to ethanol. The situation is made more complicated by the existence in yeast of a limited respiratory capacity that restricts the amount of glucose that can be respired even when the cell is exposed to a saturated oxygen environment.

The literature has also indicated that excretion of pyruvate from the cells occurs with implications for the functioning of the mitochondria in yeast. The existence of diauxic growth on ethanol implies that in some yeasts the limited respiratory capacity becomes absolute and ethanol is not respired until all the glucose is metabolised with significant implications to mitochondrial activity. Overall, however complicated the system is, CO$_2$ evolution is the key factor that represents cell metabolic functioning. CO$_2$ exit gas analysis profiles from aerobic batch cultivations of *S. cerevisiae* can be divided into 7 distinct phases on the basis of metabolic production and consumption (Locher et al., 1993).

Nevertheless the objective is to base the control of aerobic batch fermentations on events, such as achievement of a certain concentration of a product, rather than on a time basis, to achieve this control, measurement of the concentration of the nutrients and various products is required. If the required product itself can be measured on-line, enabling continual monitoring of the process yield and productivity can be carried out. On-line measurements of relevant biological variables, however, are demanding and difficult. Although on-line measurements of some fermentation variables have recently shown some success, there is still the need for reliable on-line sensors that can continuously and rapidly measure concentration of biological variables inside the fermentation broth. As indicated by this literature review, the most probable on-line parameter that would enable successful control of an aerobic yeast batch culture system is the CO$_2$ exit gas profile. The experimentation in this project was therefore directed towards demonstrating the relationship between on-line measurement of the CO$_2$ exit gas profile and aerobic yeast cell functioning, particularly metabolism and enzyme production, in aerobic batch and repeated batch cultures.
2.0 MATERIALS AND METHODS

This chapter explains the materials and methods used for performing the fermentation cultivations, the instrumentation employed to monitor the on-line variables, and also the analytical techniques utilised for measurement of the off-line variables. A process overview is provided (Figure 2.1) which illustrates the acquisition of on-line and off-line data during the fermentations.

2.1 Fermentation

Section 2.1 describes the practical aspects of the fermentation. The organism was a strain of *S. cerevisiae*; cultivation requirements of this yeast and preparations are explained in sections 2.1.1 to 2.1.4. A key feature of the work reported in this thesis was the running of the repeated batch cultures. The logistics of the timing of inoculation and sampling were studied. The details are reported in sections 2.1.3 to 2.1.6.

2.1.1 Strain selection and maintenance

*Saccharomyces cerevisiae*, strain GB4918 (DCL Yeast Ltd., Glenochil Yeast, Clackmannanshire, UK) was provide as a freeze-dried culture inside an ampoule with in a cotton wool bung in the middle of the glass ampoule.

A medium (YG) of 20% yeast extract and 6% glucose was made up and distributed into several universal bottles in 10 mL proportions. The universal bottles together with two empty 260 mL shake flasks were sterilised at 121°C, 1bar g, for 15 minutes. The ampoule was wiped with industrial methylated spirits (IMS) and gently scoured with a file where the cotton wool was placed to break open the glass. After removing the cotton wool 1 mL of the sterile medium was transferred into the ampoule to gently suspend the dry yeast which was then aseptically transferred into one of 260 mL the sterile shake flasks. Another 6 mL of the same YG medium was transferred to the yeast suspension. The shake flask was then placed in a shaker incubator at 28°C, 220 rpm for 72 hours.
Figure 2.1 Illustrates the acquisition of on-line and off-line data during the fermentations. The on-line variables are monitored every three minutes by the real-time data acquisition system (TCS unit and the mass spectrometer). Off-line samples are taken from fermentation broth for further analysis of biomass, glucose, ethanol, protein and several intracellular enzymes. The on-line and off-line data are then transferred to Excel spreadsheet for further analysis.
Julian (1991) studied the effect of storage on the kinetic performance of *S. cerevisiae* culture. This researcher reported that when samples were removed from a slope of *S. cerevisiae* (kept at 4 °C for over a year) grown in MYGP liquid medium (Table 2.4) as the inoculum to a 5 litre fermenter containing defined medium and analysed for biomass production and glucose uptake rate, the kinetic performance of the culture from the same strain changed as the slope aged. Therefore, cells did not adapt during the inoculum culture stage or lag phase in the fermenter to give a constant kinetic performance.

In order to maintain the culture in this work, after rehydrating the culture a cell line was kept on a slope of YDP agar medium (Table 2.1) as a master culture. The master culture was then subcultured onto fresh slopes every three to four months, left to grow at room temperature for three to four days and then stored at 4°C in a standard laboratory refrigerator. YDP plates (Table 2.1) were sub-cultured every week and left at room temperature to grow for 50 hours. The yeast on these plates were used to inoculate the 250 mL inoculum medium in a 1 litre shake flask to generate liquid phase cell lines in shake flasks that could then be used to inoculate the fermenter.

### 2.1.1.1 Microscopic examination of the yeast

An off-line sample from the fermenter was examined every day to determine the state of the culture, purity and viability of the cells. Visual determinations were performed using an Olympus optical microscope BH-2 (Olympus Optical Co. Ltd., UK). The most useful magnifications were at 400x (phase contrast) and at 1000x (phase contrast / oil immersion).
2.1.2 Media and reagents

The YDP agar medium used to prepare slopes and plates for all fermentations was the same and is described in section 2.1.2.1. Most fermentations were performed using a defined medium as given in section 2.1.2.2. Two of the fermentations carried out in this work were with a complex medium of MYGP described in section 2.1.2.3. MYGP complex medium was used as a different system to compare the patterns and trends of the on-line data and also to investigate the level of enzymes produce during the phases of growth with which this study was concerned. Where the fermentation was performed with a defined medium, the inoculum was also prepared with the same defined medium. In the case of MYGP complex medium the inoculum was also prepared in this complex medium.

2.1.2.1 YDP agar medium

To prepare the YDP agar plates and slopes for the purpose of subculturing \textit{S.cerevisiae}, the components listed in Table 2.1 (Oxoid Ltd., Basingstoke, Hampshire, England) were weighed and suspended in the required amount of deionised water, placed on a magnetic stirrer with a hot plate, mixed and heated. Once all the components were dissolved the medium solution was then transferred into glass autoclavable bottles. The agar medium was autoclaved at 121°C (1 bar) for 20 minutes. Once cooled to about 60°C the medium was transferred aseptically to the petri dishes and some pre-sterilized universal bottles (the bottles were positioned at an angle of some 10° to the horizontal) to set. The plates and bottles were left at room temperature for the excess moisture to dry before storing at 4°C.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td>10</td>
</tr>
<tr>
<td>Glucose</td>
<td>20</td>
</tr>
<tr>
<td>Peptone</td>
<td>20</td>
</tr>
<tr>
<td>Agar</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2.1 The solid media used for storage of \textit{Saccharomyces cerevisiae}. 

62
2.1.2.2 Defined medium

A defined medium based on defined medium of Fiechter et al. (1981) was used in this work. The medium is reported to allow aerobic growth of up to 10g/L dry biomass in a repeated batch culture (Fiechter et al., 1981). The medium had the composition (g/L) shown in Table 2.2 and 2.3. All chemicals were from Sigma (Poole, Dorset) unless stated otherwise.

Components of this medium were made up in deionised water as four different solutions and mixed together after sterilisation.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution (A)</td>
<td>23.6 g/L</td>
</tr>
<tr>
<td>Solution (B)</td>
<td>31.753 g/L</td>
</tr>
<tr>
<td>Vitamin solution (C)</td>
<td>2 ml/L</td>
</tr>
<tr>
<td>Trace salt solution (D)</td>
<td>2 ml/L</td>
</tr>
<tr>
<td>antifoam</td>
<td>0.2 ml/L</td>
</tr>
</tbody>
</table>

Table 2.2 The total concentration of defined medium used for cultivation of Saccharomyces cerevisiae (Fiechter et al., 1981).

Solutions A and B were made up separately and autoclaved (121°C, 1bar g) in different proportions. The method and the timing of the autoclave is described in section 2.1.3 and 2.1.4.

A 500 times concentrated stock solution of vitamins (D) and trace salt solution (C) was made. To avoid any precipitation, solution (C) was prepared in the order presented in Table 2.3. The solution was then acidified with H₂SO₄ to pH 2.4 and stored at room temperature. The vitamin solution was kept at -20°C for up to two months. The vitamin solution and trace salt solutions were filter sterilised into solution B at room temperature and added to the main salts of solution A.
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mineral salts solution (A)</strong></td>
<td>g/L</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>17.8</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>5.7</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Solution (B)</strong></td>
<td>g/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>30.0</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>1.2</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>0.15</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.243</td>
</tr>
<tr>
<td>meso-Inosotol</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Trace salt solution (C)</strong></td>
<td>mg/L</td>
</tr>
<tr>
<td>FeCl₃.6H₂O</td>
<td>100</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>30</td>
</tr>
<tr>
<td>MnSO₄.2H₂O</td>
<td>32</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>0.8</td>
</tr>
<tr>
<td>NaMoO₄.2H₂O</td>
<td>5</td>
</tr>
<tr>
<td>CoCl₂.6H₂O</td>
<td>5.6</td>
</tr>
<tr>
<td>H₃BO₄</td>
<td>15</td>
</tr>
<tr>
<td>KI</td>
<td>2</td>
</tr>
<tr>
<td><strong>Vitamin solution (D)</strong></td>
<td>mg/L</td>
</tr>
<tr>
<td>d-Biotin</td>
<td>0.1</td>
</tr>
<tr>
<td>Ca Panthothenate</td>
<td>2</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>15</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>4</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.3  The defined media used for cultivation of *S.cerevisiae* (Fiechter *et al.*, 1981).
2.1.2.3 MYGP undefined medium

A typical undefined chemical medium used for the growth of many organisms is Malt Extract, Yeast Extract, Glucose and Mycological Peptone (MYGP). The medium has been used for successful growth of brewing yeast by Knights (1981) based on the result of Rainbow (1970). This medium was prepared using deionised water and autoclaved (121 °C at 1 bar g).

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malt Extract</td>
<td>3.0</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>3.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>30.0</td>
</tr>
<tr>
<td>Mycological Peptone</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Table 2.4 The composition of the MYGP culture medium.

2.1.3 Inoculum preparation

For all defined medium fermentations the inoculum was prepared according to Fiechter et al., (1981) and is shown in Table 2.3. 200 mL of solution A was prepared by dissolving the ingredients in deionised water inside the inoculum flask (a 1 litre shake flask with a side arm connected to a fermenter needle). 50 mL of solution B was made by suspending the ingredients of B in deionised water inside a 260 mL shake flask. The flasks were plugged with cotton wool bungs and placed in the autoclave for 20 minutes (121°C and 1 bar g). After the flasks cooled down to room temperature, the contents of the small flask was aseptically transferred into the inoculum flask. 1ml of each 500 times concentrated solutions C and D was filter sterilised into the flask using a hypodermic syringe, 0.2 μm filter (acrodisk) and a sterile needle.

For complex medium fermentations 250 mL of the MYGP medium (Table 2.4) was prepared by dissolving the components of the medium in deionised water at room temperature. The flask was autoclaved for 20 minutes (121°C and 1 bar g).
A metal loop was sterilised in an open flame until red hot and then cooled by touching the agar briefly. Using the metal loop a small amount of yeast from the agar plate was aseptically transferred into the inoculum flask. The flask was incubated at 28 °C for 72 hours on a rotary shaker (Controlled Environment Incubator Shaker, New Brunswick Scientific Co. Inc., Edison, New Jersey, U.S.A.) running at 220 rpm. The inoculum flask was then used to inoculate the fermenter.

Inoculum size and age was adjusted so that the first off-line sample was taken at the mid exponential growth phase of the culture, some 10-12 hours after inoculation of the fermenter. For repeated batch cultures, 95% of the previous batch was emptied each time and the remaining 5% was then used as the inoculum to the next batch, unless otherwise stated. Time allowed for the set points to reach their set value was one hour (Table 2.5).

2.1.4 Fermenter preparation

The actual components of the medium are listed in Tables 2.3 and 2.4. In order to keep the composition of the medium component exactly the same for different batches of the repeated batch cultures, 20 L of the medium was made up in a 20L aspirator, 5 litres of which was used for each batch. For the single batches the same procedure was followed making up 10L of the medium and autoclaving the medium in the 20 L aspirator to keep the sterilisation the same.

The top opening of the aspirator was plugged with a silicon rubber bung. Through the silicon rubber bung two 15 cm length of 0.625 cm diameter stainless steel tubes were inserted. One of these stainless steel tubes was connected to a sterilisable filter and the other one was connected to one end of a stericonnectors (LH Fermentation Ltd., Reading, UK) via a 0.625cm silicon rubber tubing. The side arm was located towards the base of the aspirator which was stoppered with a silicon rubber bung with a 0.625 cm stainless steel tube through the bung. This end of the aspirator was connected to the fermenter via silicon rubber tubing and a stericonnectors. The aspirator was calibrated using a 1L measuring cylinder and marked up to 20L.

Solution A was prepared with deionised water and autoclaved in the 20L aspirator for 30 minutes (121 °C, 1bar g). Solution B was prepared separately with deionised water in the 2L shake flask and autoclaved for 20 minutes (121 °C, 1bar g). The vitamin solution (C) and trace salt solution (D) were filter sterilised into the 2L shake flask, at room temperature. When the aspirator had cooled to room temperature, the content of the 2L flask was added to the aspirator by gravity feed via the stericonnector by opening the gate.
clips on the silicon rubber tubing. The medium was mixed and was then ready to be pumped into the presterilised fermenter.

The fermenter ancillaries, i.e. filters, sampling device, antifoam, empty glass measuring cylinders for acid and base, with all the additional lines and needles, acid (1L of 2M H$_2$PO$_4$) and base (1L of 2M NaOH) in the 1L glass bottle containers were autoclaved for 20 minutes at 121 °C and 1bar g.

The pH and DOT probes were checked and calibrated prior to the fermenter sterilisation. The pH probe was calibrated by adjusting the pH to 4 and 7 using standard pH solutions. The DOT probe was adjusted to 100% in air and 0% by passing nitrogen gas through the probes membrane.

The fermenter was sterilised for 20-30 minutes with deionised water in situ, at 121 °C at 1bar by passing steam through the heat exchanger rings. Once the fermenter had cooled down to 28°C (by switching the temperature controller on to pass cold water through the heat exchanger) all ancillaries were connected, the fermenter was emptied under air pressure to avoid any back pressure inside the vessel leading to contamination of the fermenter (see section 2.1.5.1), fresh sterilised medium was pumped into the fermenter using a Watson-Marlow pump (Falmouth, U.K.), all set points (Table 2.5) were adjusted and the pH controller was switched on.

Both pH and DOT probes were calibrated again after sterilisation of the fermenter. The DOT probe was calibrated in the sterile medium before inoculation. A DOT of 100% was determined by allowing the probe to equilibriate in the sterile medium with an air flowrate of 2.5 L/min, agitator speed of 700 rpm and temperature of 28°C. The pH probe was recalibrated by taking a sample and checking with an independently calibrated pH probe.

pH was controlled using 2M NaOH and 2M H$_2$PO$_4$. For all fermentations, set points of the controlled variables were standard and given in Table 2.5.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Set point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agitation</td>
<td>700 rpm</td>
</tr>
<tr>
<td>Temperature</td>
<td>28 °C</td>
</tr>
<tr>
<td>pH</td>
<td>4.5 log[H$^+$]</td>
</tr>
<tr>
<td>Inlet Air Flowrate</td>
<td>2.5 L/min</td>
</tr>
</tbody>
</table>

Table 2.5 Set points of the controlled variables for all fermentations.
Finally the mass spectrometer and the data logging system were started in order to monitor all the on-line variables (Table 2.6) every three minutes. Off-line sample intervals were different for different fermentations and were usually taken every hour at the beginning and every half an hour as the exit CO$_2$ concentration approached the first peak due to the glucose consumption. Sampling is described in section 2.1.6 and sample analysis methodology is explained in section 2.3. All measured and monitored variables are indicated in Table 2.6 and shown in Figure 2.1, the process overview.

The fermenter was then ready to be inoculated with 250ml of the prepared inoculum (see section 2.1.3). Polypropylene glycerol (PPG, 2020) was added to the broth at about 0.5ml/L to prevent foaming. In repeated batch cultures addition of PPG was not necessary for other batches other than the first batch culture.

<table>
<thead>
<tr>
<th>On-line monitored variables</th>
<th>Off-line measured variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet-Outlet gases</td>
<td>Biomass (OD, Dry cell weight)</td>
</tr>
<tr>
<td>pH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Temperature</td>
<td>Glucose</td>
</tr>
<tr>
<td>Agitation</td>
<td>Protein (Total soluble)</td>
</tr>
<tr>
<td>DOT</td>
<td>Intracellular Enzymes</td>
</tr>
</tbody>
</table>

Table 2.6  Measured and monitored variables during the time course of the fermentation processes.
2.1.5 Aseptic emptying and refilling of the fermenter

After sterilisation of the fermenter with deionised water in-situ, and at the end of each batch in a repeated batch experiment, the fermenter had to be emptied and refilled with fresh sterile medium. A sample was taken from the last batch of a repeated batch culture for off-line analysis which is the inoculum to the next batch. For single batches or the first batch of a repeated batch culture, an off-line sample was taken for an OD check after inoculating the fermenter with the 250mL inoculum from the shake flask.

Aseptic emptying and refilling of the fermenter was done manually by having the fermenter under sterile air pressure to avoid any contamination of the fermenter system. The following sequence of steps were developed to ensure asepsis of the fermenter system (sections 2.1.5.1 and 2.1.5.2).

2.1.5.1 Aseptic emptying of the fermenter

1. Agitation was switched off in order to observe the volume of the broth from the volume scale on the glass fermenter. The agitation was switched back on again to prevent the yeast from settling down by mixing the broth with a slow mixing of 50-80 rpm.

2. Addition of acid or alkali to the harvest were prevented by switching the pH probe off. To avoiding over heating of temperature probe while the tank was empty, the temperature probe was also switched off.

3. A sterile air was flow maintained to the fermenter at the same rate as during the fermentation, 2.5L/min, to maintain a slight over pressure.

4. The outlet air filter valve was closed while monitoring the pressure inside the vessel from the gauge, before the condenser outlet air, to be less than 0.5 bar.

5. The broth was emptied from the sampling point into a one litre measuring cylinder, leaving 250 mL of the yeast suspension inside the fermenter vessel. Sterile air was still flowing into the fermenter.

6. The outlet air filter valve was then slowly opened.
2.1.5.2 Aseptic refilling and self inoculation of the fermenter

Once the fermenter was empty of liquid while maintaining the sterile air flow to the vessel, the following procedure was employed:

1. Fresh sterile medium was pumped into the fermenter by a Watson-Marlow (Falmouth, U.K.) pump with a flow rate of 225 mL/min.

2. The pH probe and temperature probes were switched on again with the same set points as before, namely, pH 4.5 and temperature 28 °C. Agitation was slowly increased manually to above 700 rpm and switched to automatic control with a set point of 700 rpm.

3. After about 45-60 min once the variables reached the set point values the broth was ready for off-line sampling.

On-line variables were monitored during the refill and emptying the vessel. The exact timing of the refill can be observed from on-line data.

2.1.6 Off-line Sampling

Samples of 60mL to 80mL were aseptically removed from the fermenter (figure 2.1). Samples were put on ice immediately after being removed from the fermenter to slow down the metabolic activity of the cells. 40 mL of the sample was disrupted with a Micron Lab-40 homogeniser (1200 bar, 2 passes) immediately. The homogenate was used for further analysis of protein and several intracellular enzymes. 20 mL of the sample was used for dry cell weight determination (Section 2.3.1.1). About 10 ml of the sample was filtered and stored at -20°C for later HPLC analysis of glucose and ethanol (Section 2.3.2). The remaining proportion of the sample broth was used for optical density measurements (Section 2.3.1.2). Off-line sample intervals are as stated for each individual fermentation (Chapter 3).

The first off-line sample from the fermenter was used to check the calibration of the pH probe and to examine the purity of the culture, microscopically.
2.2 Instrumentation

This section outlines the equipment and instrumentation used for fermentation, on-line data acquisition, cell disruption, HPLC analysis and also the computing equipment used to process data.

2.2.1 Fermenter equipment and instrumentation

The fermenter used was a LH 2000, 7L glass fermenter, (LH Fermentation, Reading, England) with a working volume of 5L. The fermenter has three equally spaced six bladed Rushton disc turbines and four tank baffles. Fig.2.2 shows a schematic diagram of the fermenter including the top plate with port allocation.

The following components of the fermenter are identified:
- **a: Top Plate**
- **b: Sample Line**
- **c: One of the three Rushton turbines**
- **d: Backing Flange**
- **e: Leg**
- **f: Nut and Washer**
- **g: Impeller Shaft**
- **h: Air Sparger (positioned to sparge air below the lowest turbine on the impeller shaft)**
- **i: Heat exchanger**
- **j: Bottom Plate**
- **k: Nut and Washer**

The following ports are identified on the top plate for sampling, inoculation, air input, acid, alkaline and antifoam additions, pH and DOT probes:
1. **Phosphoric Acid**
2. **pH Probe**
3. **DOT Probe**
4. **Sterile Air into the Fermenter**
5. Three way needle used to expand a single port to three independent ports for introducing (antifoam, 2M NaOH, and sterile medium into the fermenter)
6. **Sample port**
7. **Exit gas into the mass spectrometer through the condenser**
8. **Inoculum flask**
Figure 2.2 Diagram of the LH 7L fermenter used for all fermentations in this work. The working volume of 5L was chosen for the fermentations.
The fermenter was instrumented with a TCS 6358, eight loop PID controller (Turnbull Control System Ltd., Worthing, UK.) for converting the analog to digital signals. The TCS unit was communicating with the data monitoring and control system (section 2.2.3) via a serial line.

The pH was measured by a steam sterilisable Ingold 465 (Ingold, Urdorf, Switzerland) probe. The probe was directly connected to the controller. At a given set point, the controller activates the peristaltic pumps for further addition of acid or alkali from the respective reservoirs to the fermenter.

Dissolved oxygen transfer was measured using an Ingold, steam sterilisable polographic DOT probe (Ingold, Urdorf, Switzerland).

Temperature was measured by a resistance thermometer and controlled by a 500W cartridge heater or the cooling water supplied through the heat exchanger for consecutive cooling or heating of the system.

Temperature and pH of the broth, inlet gas into the fermenter and stirrer speed were monitored and controlled under set points (Table 2.5), other variables such as dissolved oxygen tension (DOT), and load cells measuring alkali and acid addition to the broth were measured variables which were only monitored. All these variables were then transmitted to the data logging system (Bio-I or RT-DAS).

2.2.2 Exit gas analysis

The relative amounts of $N_2$, $O_2$, $CO_2$ and Ar in each inlet and outlet gas streams were measured by a 32 channel VG MM8-80, magnetic sector mass spectrometer (VG Ltd., Middlewich, England). The frequency at which analysis was available depended on the number of users on the system at the time. Analysis were usually available every three minutes. Calibration of the mass spectrometer was automatically done every twenty four hours.

Measurements from the mass spectrometer were transferred to an IBM PC which were in turn transferred to the available data logging system (RT-DAS).
2.2.3 Data acquisition and processing

Bio-i (BCS Ltd., Maidenhead, Kent, England) and RT-DAS (Real Time Data Acquisition System, Surrey), are the two data monitoring and control systems used for collection, storage and graphical presentation of the on-line data from the TCS unit and the mass spectrometer. The on-line measurements were recorded every three minutes.

On-line data could then be presented numerically or graphically at any time during the fermentation operation. All measurements were then saved on floppy disks to be transferred into a Macintosh computer and/or the Sun workstation for later analysis and correlations of the on-line data with the off-line measurements from fermentation samples.

2.2.4 Micron Lab 40 homogeniser

Samples were disrupted within 2 minutes of being removed from the fermenter. A Gaulin Micron Lab 40 homogeniser (APV, Mecklenburger Strasse 223, D-2400 Lubeck 14, Germany) was used to disrupt the cells at 1200 bar by two passes (experimentation showed that these conditions gave maximal protein release irrespective of growth phase). The operating pressure can be set in the range of 100 to 1600 bar by means of a potentiometer which is on the digital display. The sample chamber is maintained at 0°C to minimise sample heating during cell disruption. Adequate cooling to the cell suspension was provided by turning the glycol on to the cooling coils one hour prior to using the homogeniser. The product cylinder was filled up with 40 mL of the cell suspension (section 2.1.6). The cell suspension level must not be below 6mm from the top of the product cylinder to prevent the penetration of solid materials, impurities and air inclusions. Having all parts including the valve housing and the cover centred, the homogenisation step could be started. The process was automated and was started by depressing the Start-up Automatic operation button. The valve housing and the product cylinder were thoroughly rinsed with deionised water before use. The homogenate was then used to analyse the total protein and the intracellular enzymes of the off-line samples.
2.2.5 High performance liquid chromatography (HPLC)

A HPLC system was used for quantitative analysis of ethanol, glucose and pyruvate. Using a LDC Milton Roy isocratic HPLC pump (model Constametric III), the sample was pumped through the autosampler (LDC Milton Roy autosampler, model LC 241) into the column to the UV detector (LDC Milton Roy ultraviolet detector, model spectromonitor III) and then the RI detector (LDC Milton Roy refractive index detector, model RefractoMonitor IV) before discharge to the waste reservoir.

The column used for initial analysis was a 15 cm long fermentation monitoring column (Biorad), protected by a Biorad cation H\textsuperscript{+} guard column. The mobile phase was 0.004 M H\textsubscript{2}SO\textsubscript{4} (HPLC grade H\textsubscript{2}SO\textsubscript{4} obtained from Fisons). At a flow rate of 0.65 mL.min\textsuperscript{-1}, the retention times of ethanol, glucose, and pyruvate were 10.6 minutes, 5 minutes, and 5.5 minutes respectively. The operating conditions for both columns were the same. The column was kept in a water bath at 50\textdegree C and UV detection was at 210 nm for the pyruvate analysis. Refractive index was used for detection of glucose and ethanol. For a number of fermentations, the observation was made that better resolution was required for ethanol assays (Chapter 3). Therefore an Aminex HPX-87H 30cm column was used. This column gave better resolution between the components, however a longer time was required for analysis of the samples (at a flow rate of 0.7 mL.min\textsuperscript{-1}, retention time was 17 minutes for ethanol and 8 minutes for glucose). A range of known concentrations of ethanol, glucose and pyruvate were used for calibration. The calibration curve for all analytes were linear over the range tested (between 0.5-30 g/L glucose, 0.25-15 g/L ethanol, and 0.033-2 g/L for pyruvate).

The HPLC analysis software used was Perkin Elmer Nelson 2100 which was running on a personal computer, IBM model 55 SX.
2.3 Off-line analytical techniques

Fermentation samples were removed aseptically from the fermenter as described in section 2.1.6. Samples were then analysed to determine the biomass, ethanol and glucose content of the broth. Some intracellular enzymes, and the total protein concentrations were also analysed after homogenising some 40 mL of the sample.

2.3.1 Biomass Determination

Sonnleitner et al., (1992) reviewed different methods of biomass determination. For the work in this project, biomass concentration of the fermentation broth was determined by two different methods (i) measuring the dry cell weight concentration of the broth and (ii) determining the optical density of the broth.

2.3.1.1 Dry cell weight measurement

The dry weight measurements were obtained by filtering 20 mL samples through a predried (24 hours in a drying oven at 85°C) glass microfibre filter (Whatman, grade GF/C, 0.45 μm pore size, 4.7 cm diameter) positioned on a Sartorious vacuum filter apparatus (Sartorious Instruments Ltd., Belmont, England). A vacuum was applied to remove the liquid component of the sample, leaving the broth solids. The solid residual was then rinsed with 10 mL of deionised water which was also removed by reapplying the vacuum. The solid residue and the paper were placed in an 85°C oven for 24 hours to dry. The filter and dry biomass were placed in a desiccator to cool to room temperature and reweighed. The difference between the weight of the filter paper before and after the addition of the residual solids to the filter paper was used to determine the dry weight of the solids in the 20 mL sample. Therefore the biomass concentration could be calculated in grams of dry cell per litre of culture broth at a given sample time.
2.3.1.2 Optical density measurements

Fermentation broth was diluted with deionised water to obtain an optical density reading less than 0.4 units at 660 nm (Appendix 1), with a Pye Unicam single beam SP6-550 spectrophotometer against deionised water as a blank. The biomass concentration was expressed in grams of biomass per litre of broth by correlating the optical density readings with dry cell weight measurements.

2.3.2 Glucose and ethanol measurements

Glucose and ethanol concentrations were measured using a high performance liquid chromatography system. For some fermentations pyruvate concentration of the same samples has also been measured. 10 mL samples from the fermentation broth were filtered through a GFA standard glass micro fibre filter (0.45 μm pore size, 25 mm diameter) with a 25 mm polycarbonate syringe filter holder (Sartorious, Longmead, Surrey). The filtrate was stored in 7mL bijou bottles at -20°C for later HPLC analysis of glucose and ethanol. The frozen samples were thawed at room temperature for HPLC analysis of ethanol, glucose and pyruvate.

2.3.3 Total protein

After homogenisation, soluble protein was determined by the Bradford Coomassie Brilliant Blue dye (G250) binding method (1976). The reagent was calibrated in the range 0-100 μg mL\(^{-1}\) using Bovine serum albumin (BSA, BDH). 100 μL aliquots of the samples were diluted as required with K\(_2\)HPO\(_4\) (100 mM, pH 6.5), added to 2.9mL of Bradford reagent (Biorad), and read against the reagent blank (595 nm) after 5 minutes. A Beckman DU64 spectrophotometer (Beckman, High Wycombe, Bucks.) was used for the total protein assays.
2.3.4 Enzyme Assays

After homogenising the cells, enzyme analysis was performed as soon as possible using a Beckman DU64 spectrophotometer (Beckman, High Wycombe, Bucks.) with a Kinetic Pac accessory. Linear regression was performed on the rate of absorbance change and \( R^2 \) values of less than 0.98 were rejected. All enzyme assays were completed within twenty minutes of sampling. The homogenate was kept on ice. All chemicals were from Sigma (Poole, Dorset) unless otherwise stated.

The enzyme activities in units per litre of the sample broth was calculated from (Bergmeyer, 1983):

\[
\text{ADH units of activity/mL} = \frac{\Delta A \, \text{(min)}^{-1}}{6.2} \left( \frac{V_t}{V_s} \right) (D)
\]

where:

- \( V_t \) is the total volume of assay mixture and sample in the cuvette
- \( V_s \) is the sample size in the cuvette
- \( \Delta A \) is the rate of change of absorbance, and 6.2 (mole L\(^{-1}\) cm\(^{-1}\)) is the molar extinction coefficient, and D is the dilution factor.
**Alcohol Dehydrogenase**

Alcohol: NAD$^+$ oxidoreductase (EC 1.1.1.1)

**Reaction:**

\[
\text{Ethanol} + \text{NAD}^+ \xrightleftharpoons{\text{ADH}} \text{acetaldehyde} + \text{NADH} + \text{H}^+ 
\]

The formation of acetaldehyde from ethanol may be monitored by measuring the increase in the absorbance which is due to the formation of NADH (Bergmeyer 1983).

**Reaction Mixture:**

The assay mixture was prepared as follows:

- 17.5 mL Ethanol (absolute)
- 9.0 mL NAD$^+$ (0.1 M)
- 3.1 mL Semicarbazide HCL (1.0 M)
- 5.0 mL Glutathione reduced (0.1 M)
- 465.4 mL Tris buffer (0.05M, pH 8.8)

The solution was made up to 500 ml with deionised water and stored at -20°C.

**Procedure:**

Sample (50 μL) was mixed with assay mixture (3 mL, 25°C), and the rate of absorbance change recorded (340 nm) for three minutes at ten second intervals. The sample was diluted (KH$_2$PO$_4$ buffer 100mM, pH 6.5) as required. One unit of enzyme activity reduces 1μmole of NAD$^+$ per minute at 25°C.
**Glucose-6-Phosphate Dehydrogenase**

D-Glucose-6-phosphate: NADP⁺ oxidoreductase (EC 1.1.1.49).

**Reaction:**

\[
\text{Glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{6-phosphogluconolactone} + \text{NADPH} + \text{H}^+ 
\]

The oxidation reaction is favoured and the rate of increase in absorbance is a measure of G6PDH activity (Bergmeyer, 1983).

**Reaction Mixture:**
The assay mixture was prepared as follows:

- 10 mL Tris/HCl (0.5 mM, pH 7.5)
- 10 mL Glucose-6-phosphate (3.3 mM)
- 10 mL MgCl₂ (BDH, 0.63 mM)
- 2.9mL NADP⁺ (BDH, 3.8 mM)

The solution was made up to 100mL with deionised water and stored at -20°C in 50 mL aliquots.

**Procedure:**
Sample (homogenised, 200 µL) was mixed with assay mixture (3 mL, 25°C); the rate of absorbance change was recorded at 340 nm for two minutes at 10 second intervals. One unit of enzyme activity reduces 1 µmol of NADP⁺ per minute at 25°C.
Malate Dehydrogenase

L-Malate: NAD⁺ Oxidoreductase (E.C. 1.1.1.37)

Reaction:

\[ \text{Oxidoacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}^+ \]

The amount of oxaloacetate reacting per unit time, measured by the decrease in absorbance due to the oxidation of NADH is a measure of the catalytic activity of MDH.

Reaction Mixture:

The assay mixture was prepared as follows:

- 10 mL NADH (0.3 mM)
- 10 mL Oxaloacetic acid (1.51 mM)

Oxaloacetate and NADH solutions were prepared fresh and stored on ice throughout sampling and analysis. Assay mixture consisted of NADH solution (10 µL), oxaloacetic acid solution (10 µL) and 930 µL of the phosphate buffer KH₂PO₄ (0.1M, pH 6.5, 25°C).

Procedure:
Sample (50 µL) was mixed with assay mixture (950 µL) and the decrease in the rate of absorbance change recorded (340 nm) for four minutes at ten second intervals. One unit of enzyme activity oxidises 1µmole of NADH per minute at 25°C (Darley-Usmar et al., 1987).
**Hexokinase**

ATP D-Hexose 6-phosphotransferase (E.C 2.7.1.1)

**Reaction:**

\[
\text{D-Glucose} + \text{ATP} \xleftrightarrow{\text{Hexokinase}} \text{D-Glucose-6-P} + \text{ADP}
\]

\[
\text{D-Glucose-6-P} + \text{NADP}^{+} \xrightarrow{\text{G6PDH}} \text{D- Gluconate-6-P} + \text{NADPH} + \text{H}^{+}
\]

This assay is a coupled reaction, the amount of hexokinase reacting per unit time is indirectly measured by the rate of increase of absorbance which is a measure of G6PDH activity to oxidise the NADP⁺ in the second reaction.

**Reaction mixture:**
Three different solutions for the assay mixture were prepared as follows:

a) Solution (a) consisted of 5 g Glucose (0.5 M), 1.27 g MgCl₂ (8 mM), and 0.5 g NADP⁺ (0.91 mM) dissolved in 50 mL of Tris buffer (50 mM pH 7.5) as described for G6PDH assay (section 2.3.3.2.2). This solution was stored at -20 °C.

b) 1.0 mL Adenosine triphosphate (0.65 mM). The solution was made fresh and stored on ice.

c) 0.71 mL Glucose-6-phosphate dehydrogenase (6.3 x 10⁻³ UmL⁻¹). The solution was made fresh and stored on ice.

**Procedure:**
One mL of solution (a) (25°C) was added to the ATP solution (100 μL) and G-6-PDH (10 μL). Sample (10 μL) was added to the assay mixture and the rate of absorbance increase recorded (340 nm) for 8 minutes at 20 second intervals, the sample was diluted as required with Tris buffer. One unit of enzyme activity reduces 1 μ mole of NADP⁺ per minute at 25°C.
2.4 Summary

In this chapter materials and methods used to carry out the fermentations for this project are described. The equipment used for the fermentations and on-line data acquisitions are outlined, and the analytical techniques for processing off-line samples from fermentations are explained. Equipment used for the project was found to be satisfactory and were not modified in any form.

Methods described in this chapter were used to perform a series of batch and repeated batch fermentations. Experimental techniques and fermentation conditions used for all fermentations were the same. On-line data were recorded every three minutes from inoculation time to the end of growth on ethanol (Figure 2.1). Off-line samples from the fermenter were analysed for optical density, dry cell weight, ethanol, and protein concentrations and intracellular enzymes such as ADH, MDH, G6PDH and hexokinase as described in this chapter. Basic data from the experiments are shown in appendices 2 to 6 and analysis of the fermentation data are discussed in the next chapter. Summary statistics of the replicate assays are shown in Table 4.1, chapter 4 to demonstrate data accuracy and sensitivity of the analysis technique.
3.0 FERMENTATION DATA ANALYSIS - RESULTS and DISCUSSION

Introduction

On-line monitored variables and off-line measured variables were obtained from a series of batch and repeated batch cultures to identify the profiles of these variables during the time period of the cultivations. Reproducibility of these profiles were then investigated. The possibility of using readily available on-line monitored data for predicting events (such as product formation or timing of the seed transfer) and making decisions on fermentation processes were examined.

All fermentations were carried out with the same strain of yeast using the same fermentation techniques (e.g. inoculum preparation, sterilisation) and conditions (temperature, agitation, pH) (see chapter 2, section 2.1.4). Experiments were designed to:

1) Establish correlations between the on-line and off-line data.

2) Study reproducibility of any correlations.

3) Reduce batch to batch variability.

In this chapter, fermentation results are divided into three sections based on the type of fermentations (single or repeated batch culture, see chapter 2, section 2.1.4) and growth media used (defined or complex, see materials and methods, chapter 2, section 2.1.3).

Section 3.1 includes the single batches of S.cerevisiae fermentation with defined medium. Single batch cultures, SBD1, SBD2, SBD3, and SBD4 were performed. Cultivation results and data correlations for batches SBD1, SBD2, and SBD3 are presented in appendix 2: Single batch SBD4 is shown to investigate the relationship between the on-line CO₂ exit gas analysis and DOT data with the metabolic changes in profiles of intracellular enzymes (section 3.1.2). The reproducibility of the off-line analysis profiles of these cultures, based on the first CO₂ exit gas analysis peak, are then discussed in section 3.1.3.
Section 3.2 includes the repeated batch cultures of *S. cerevisiae* with defined medium. A series of preliminary repeated batch cultures was carried out to examine the reproducibility of the on-line data, the initial inoculum volume required for the fermentations and the timing of inoculation for off-line sample analysis of the required phases under investigation (section 3.2.1). To reduce the batch to batch variability observed with the single batch experiments, repeated batch cultures RBD1, RBD2, and RBD3 were performed. On-line and off-line data correlations for these experiments are presented in appendices 3, 4, 5. The reproducibility of the off-line analysis profiles of fermentations RBD2 and RBD3, based on the first CO$_2$ exit gas analysis peak, are then discussed in section 3.2.2.

Defined medium fermentations used to investigate the reproducibility of single and repeated batch cultures are presented in Table 3.1 as a matrix of data. Fermentation RBD2-1 is also included in single batch comparison (see section 3.1.3).

<table>
<thead>
<tr>
<th>Batch Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBD1</td>
</tr>
<tr>
<td>SBD4</td>
</tr>
<tr>
<td>RBD2-1</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Table 3.1 Fermentation experiments using defined medium. SBD are the single batch experiments carried out with defined medium. RDB are repeated batch cultures carried out with the same defined medium.

Section 3.3 includes the cultivation of *S. cerevisiae* in complex medium under the same fermentation conditions used for the defined medium fermentations. Complex medium fermentations were used to compare the enzyme activity levels and the profiles of on-line CO$_2$ exit gas analysis data of cultures grown on defined medium to fermentations with complex medium.

Off-line measurement variability analysis for the results presented in this chapter is discussed in chapter 4.
3.1 Single batch cultures of *S. cerevisiae*

Single batch fermentations using defined medium were performed to investigate the changes in the underlying metabolic profiles of the enzymes of central carbon metabolism and the relationship of these profiles with readily identifiable events, such as the first CO$_2$ peak of the exit gas analysis.

On-line variables of the fermentations were obtained from the real time data acquisition system (chapter 2, section 2.2.3). CO$_2$ exit gas analysis showed a similar trend to that reported by Locher *et al.* (1993, a), although in that study the researchers were particularly concerned with the interpretation of exit CO$_2$ profiles and the production of extracellular metabolites. This interpretation has been extended in this thesis to include intracellular enzyme activities which correlate with the exit CO$_2$ profile and the extracellular metabolites. Intracellular enzymes such as G6PDH, hexokinase, ADH, and MDH were chosen as model products and metabolic indicators of the transition phase on which the operational decision making was based on.

Four single batch experiments SBD1, SBD2, SBD3, and SBD4 were carried out (section 3.2.1) as individual experiments to investigate the correlation of on-line CO$_2$ and DOT data and the off-line data of some intracellular enzymes. The results of these experiments were then used to indicate the reproducibility of the enzyme profiles and their correlation with the CO$_2$ exit gas profile in section 3.1.2.

DOT and CO$_2$ exit gas profile data of all experiments in this section were monitored from inoculation time of the fermentation to the end of phase 7 of the CO$_2$ exit gas analysis. Off-line samples were analysed from the middle of phase 1 to phase 4 of the CO$_2$ exit gas analysis which corresponds to the middle of exponential growth phase to the early slow growth phase of the biomass. Experimental data presented in the graphs are from the starting time of the first off-line sample taken from the fermenter.
3.1.1 CO2 exit gas analysis

Locher et al. (1993, a) divided the aerobic fermentation into seven phases depending on the profile of CO2 exit gas concentration and the phases were related to apparent carbon source at that time point in the fermentation. For ease of interpretation, a similar phase numbering system is used for this work. Figure 3.1 shows a typical profile of a single batch experiment 'SBD2'. The CO2 exit gas profile, indicated full consumption of glucose at about 21 hours. Glucose is metabolised into ethanol, biomass, and CO2 in phase 1 and phase 2. Subsequent growth then occurred on ethanol which had been produced during growth on glucose. Phases 3 and 4 were characterised by consumption of organic acids, namely pyruvate and acetate. The metabolic background for phase 5 was reported (Locher et al., 1993 a) to be unknown. During phase 6 ethanol is metabolised and acetic acid is accumulated which is utilised later in phase 7, indicated by a sharp peak in the CO2 exit gas profile. Ethanol and any other carbon sources are depleted from the medium in phase 7.

![Figure 3.1](image_url)

Figure 3.1 %CO2 exit gas profile of single batch 'SBD2' from inoculation time to the end of growth on ethanol. Similar phases reported by Locher et al. (1993, a) are presented in Figure 1.8.
3.1.2 Correlation (relationship) of intracellular enzyme activities with CO₂ exit gas profiles in single batch fermentations

To investigate the correlation of on-line and the off-line data obtained from aerobic batch cultures of *Saccharomyces cerevisiae* grown in defined medium (chapter 2, section 2.1.2.2), single batch fermentations SBD1, SBD2, SBD3, and SBD4 were carried out. Phases 1 to 4 were chosen to study the events at the first peak of the CO₂ exit gas analysis since the first peak was chosen as the on-line phase recognition signal. The investigations would then assist the decision making process.

Although the phases of interest were phase 1 to phase 4 of CO₂ exit gas analysis, fermentation SBD4 was sampled from mid exponential growth phase of biomass to the end of ethanol consumption by the biomass which is referred to as phases 1 to 7 of the CO₂ exit gas profile. SBD4 is presented in this section as an example of the single batch experiments to show the correlations of on-line monitored and off-line measured variables in a batch fermentation. Experimental results for SBD1, SBD2, and SBD3 are presented in appendices 2.1, 2.2, and 2.3. The patterns of the profiles for single batch fermentations with defined medium presented in appendix 2 are the same as those of SBD4 in this section, unless stated otherwise.

Figure 3.2-a illustrates the CO₂ exit gas analysis and the DOT monitored variables from 14 hours at phase 1 to the end of phase 6. Phase 7 was noted at 41 hours. Off-line samples are shown from 14 hours to 42 hours which is associated with phase 1 to the last sample after phase 7.

Figures 3.2a-j show the on-line, and off-line profiles of the monitored and measured fermentation variables of SBD4. The figures show many key observation, which are highlighted in the figure captions. Presentation of these typical figures are followed by more detailed discussion in this section. The dashed lines between the points on the enzyme activity graphs help to indicate which measurement set the points belong to. There is no implication that the underlying trend represented by the data is piece wise linear.

Enzyme activities can be expressed as U/mL of the culture as reported by Maitra and Lobo (1971), Steube and Spohn (1994), Salmon and Buckland (1992), Ahlmann et al. (1986),
and Blankenstein and Kula (1991). Some authors have used U/g of dry cell weight to express enzyme activity levels in their work (Schaaff et al., 1989, Pascual et al. 1988, and Gowda et al. 1988). Measurements of enzymes in U/mg of total protein has also been reported (Polakis and Bartley, 1965 and Postma et al., 1989). Figures 3.2e-j show the intracellular enzyme activities expressed as U/mL of the fermenter sample, U/g of dry cell weight, and U/mg of total protein concentration to express the profiles of these enzymes before and after the CO₂ peak. The U/mL profiles (Figures 3.2-e,f) give less variability since introducing dry cell weight or protein concentrations into the unit activity measurements causes propagation of measurement error which resulted in greater variations in the profiles of the enzymes. The U/mL exhibits the profiles of the activity within the fermenter at a particular time whereas the U/g indicates the activity within the cell.

Phase 1.

For SBD4 (Figures 3.2 e-f), phase 1 can be observed up to 22 hours cultivation. This phase corresponds to the first exponential growth phase of biomass on glucose (figures 3.2-b and 3.2-c). The primary metabolites ethanol and pyruvic acid were being produced in this phase. At the end of phase 1 the glucose concentration had decreased to 5.4 g/L and an accumulation of ethanol was observed (Figure 3.2-b). This accumulation represents the reduction of acetaldehyde to ethanol during glycolysis (Chapter 1, Figure 1.3). The pyruvate concentration was at a 0.06 g/L, the dry cell weight concentration had reached 2.95 g/L, and the total protein concentration of the sample followed the same profile as the biomass concentration for SBD1, SBD2, and SBD3 (Appendix 2.1, 2.2, and 2.3), throughout the different phases. The phases of biomass growth on the two main substrates of glucose and ethanol are clearly seen in figure 3.2-c.

The intracellular enzyme levels are shown in figures 3.2,e-g. As the glucose was consumed in this phase, the concentration of ethanol increased. Figure 3.2-d shows an increase in the alcohol dehydrogenase (ADH) activity level throughout phase 1 of the batch in accordance with the ethanol concentration accumulation. This observation can be explained by the limited respiratory capacity (Kappeli 1986, Figure 1.5). Glucose flux was supercritical in this phase. The substrate flow exceed the respiratory capacity and the residual part of glucose that could not pass the bottleneck was metabolised reductively, acetaldehyde being reduced to ethanol, ADH was released in the cell, and ethanol was excreted into the medium. If the bottleneck is filled with glucose, ethanol remains unused and the ethanol concentration would increase in the medium.
Malate dehydrogenase activity was essentially negligible in the first phase. Polakis and Bartley, (1965) referred to MDH as the most active enzyme of TCA cycle at all stages of growth and Beck et al., (1968) reported the repression of this enzyme during growth on glucose. The inactivation of this enzyme by glucose has been reported to start 5-10 minutes after the addition of glucose to cells growing on non-fermentable carbon sources and the activity of this enzyme has been reported to diminish within 1 hour (Holzer, 1976).

Glucose-6-phosphate dehydrogenase activity increased as the biomass concentration rises in phase 1, which is consistent with glycolysis activity. Hexokinase activity level was similar to that of G6PDH activity. At 21 hours a sudden decrease of hexokinase activity was observed in phase 1. This change could be due to the inhibition of hexokinase which is caused by inhibition of phosphofructokinase activity (Stryer, 1988). Phosphofructokinase catalyses is the first irreversible reaction of the glycolytic pathway (chapter 1, Figure 1.2) and can be blocked by high levels of ATP H+, citrate, and early intermediates of the citric acid cycle. Inhibition of phosphofructokinase activity causes the accumulation of fructose-6-phosphate and glucose-6-phosphate. Accumulation of glucose-6-phosphate has been reported to suppress the glucose uptake mechanism (Sols et al., 1971) which was indicated by the parallel reduction in ADH enzyme activity at 21 hours. The accumulated glucose-6-phosphate was then oxidised by the pentose phosphate pathway to generate NADPH (chapter 1, Figure 1.3). At 23 hours, in phase 2, the inhibition was terminated and both hexokinase and ADH enzymes showed higher activity levels.

Phase 1 was about four hours shorter for SBD1 (17.75 h) and SBD2 (21 h) than SBD3 (17 h) and SBD4 (22.1 h) fermentations. This time change was probably due to the inoculum age difference between these experiments. The 250 mL inoculum for SBD2 and SBD4 was 24 hours older than the inoculum for SBD1 and SBD3.

Phase 2.
Growth phase 2 as described by Locher et al. (1993, a) was not clearly observed in SBD4. However, SBD1, SBD2, and SBD3 (Appendix 2), showed clear indication of phase 2. Better presentation of phase 2 in SBD4 may be obtained by plotting phases 1 to 4 of the CO₂ exit gas analysis in this fermentation. Locher et al. (1993, a) reported the absence of phase 2 when yeast extract was added or if the fermenter was stirred less vigorously. The conditions employed in SBD fermentations would give a well mixed fermenter yet the
phase was not easily recognisable, indicating that the high nitrogen content of the medium was the likely cause for difficulty in recognition of this particular phase. For fermentations in this project, phase 2 is from the time when the CO$_2$ exit gas concentration has stopped increasing exponentially to the beginning of the second CO$_2$ exit gas concentration peak. The time taken for phase 2 in SBD fermentations was about two hours (SBD1, 2.2 hours, SBD2, 1.9 hours, SBD3, 2.2 hours, and SBD4, 1.9 hours).

Ethanol was still being produced, pyruvate was at the maximum concentration of 0.1 g/L, glucose concentration was reduced to 0.3 g/L, dry cell weight concentration had reached 4.1 g/L at the end of phase 2. The total protein concentration of the samples followed the same profile as the biomass concentration throughout the different phases. This is also indicated for SBD1, SBD2, and SBD3 (Appendix 2.1, 2.2, and 2.3). Pyruvate started being utilised as soon as the glucose was completely exhausted. Some of this consumed pyruvate was metabolised into ethanol.

For the intracellular activity levels (Figures 3.2,e-j), maximum change was observed with MDH, a TCA cycle enzyme. MDH hardly showed any activity in the previous phase and activity started in phase 2. MDH is derepressed due to the direction of the metabolic flow. As glucose is exhausted the TCA cycle is activated and MDH is produced. ADH enzyme activity level (U/mL) had also increased in this phase. Other single batch fermentation analysis showed the same profiles of intracellular enzymes at this phase (Appendix 2.1, 2.2, and 2.3). ADH activity level U/mg of protein and U/mg of biomass indicated an increase in the activity levels in this phase which stabilised from this phase to the end of ethanol depletion (Figure 3.2-g,i). The exponential increase in the activity level of G6PDH and hexokinase were both terminated at the end of this phase. The profiles of these two enzymes were consistent with the profile of biomass production in phases 1, 2 and 3.

**Phase 3.**
Phase 3 refers to as the second peak of the CO$_2$ exit gas analysis profile. This peak is clearly observable in figure 3.2-a and can also be seen in the DOT trace. The second peak is an indication of the pyruvate uptake after the total exhaustion of glucose in phase 2. Pyruvic acid is utilised as soon as the glucose is depleted in phase 2. Glucose concentration had decreased to 0.25 g/L and the ethanol concentration was getting close to its maximum value of 8.62 g/L in phase 3. The exponential growth of biomass was
terminated and slow growth began on the accumulated metabolised carbon source, ethanol. Total protein followed the same profile as the biomass throughout the phases.

Figure 3.3-g,i shows a slight decrease in the ADH and MDH activity levels while G6PDH and hexokinase activity levels have increased in phase 3. Since the glucose was totally used and TCA cycle was active in this phase, the enzymes of the TCA cycle including MDH would be derepressed and as a consequence, the activity of all the enzymes would indicate a decreased in this phase in accordance with total soluble protein concentration (Figures 3.2-i,j). Due to the limited amount of pyruvic acid present (Figure 3.2-b), phase 3 was very short. The duration of this phase was one hour for all fermentations presented in this section.

**Phase 4.**
Phase 4 is shown by a gradual increase in the CO$_2$ exit gas concentration which was from 25 hours cultivation to about 35 hours for SBD4 (Figure 3.2-a). Locher *et al.* (1993) described this phase as growth on acetic acid, glycerol and a small amount of propionic acid (approx. 30 mg/L) and demonstrated the excretion of acetic acid in early phases of the batch. The authors also stated that the accumulation of acetic acid was until the start of phase four when the molecule reconsumed. This compound was reported to be excreted again in phases 5 and 6 by the utilisation of ethanol. The concentration of acetic acid and propionic acid was also reported to inhibit growth at concentrations of 3 g/L. Inhibition effect for pyruvic acid was shown to be above 4 g/L. The maximum level of pyruvate for experiments in this work was 0.14 g/L. In the case of SBD4, an increase in pyruvate level at 29 hours indicated that the rate of ethanol conversion to acetaldehyde and pyruvate was greater than the rate of conversion of pyruvate to acetyl-co A (see chapter 1, Figure 1.3).

However, from figure 3.2-b during phase 4 growth appeared to be predominantly on ethanol as shown by the decreasing concentration of this compound. Therefore, in figures 3.2-a and 3.2-b there is some difficulty to clearly establish all the substrates of utilisation. The presence of other phases later in the fermentation indicated that there was some change in the metabolic pool but this change could not be clearly identified. Total protein concentration (mg/mL) inside the fermenter was increasing as was the biomass concentration.
During phase 4, ADH and hexokinase activity (U/mL) increased only slightly. The levels of MDH and glucose-6-phosphate dehydrogenase (U/mL), on the other hand, increased more noticeably throughout this phase. Activity of all the enzymes with respect to biomass is almost constant (Figure 3.2-g,h).

**Phase 5.**
Phase 5 is distinguished by a small peak in the CO₂ exit gas analysis profile, and can also be seen in the DOT trace. Ethanol was still being used, ADH and G6PDH activity levels show a decrease in level, whereas MDH and hexokinase levels were increasing, as shown in figures 3.2-e and 3.2-f. Accumulation of acetic acid and reutilisation of glycerol was reported by Locher et al. (1993) in this phase.

**Phase 6.**
Phase 6 is characterised by full consumption of the ethanol. Locher et al. (1993, a) also reported the utilisation of glycerol and production of acetic acid in this phase. The presence of the sixth phase of growth can be observed in figure 3.2-a which indicates complete utilisation of ethanol (Figure 3.2-b). The second exponential increase of biomass and total protein that had started at the end of phase 4 was terminated in this phase.

There was an increase in the activity of MDH, ADH, and hexokinase activity (U/mL) throughout this phase. A significant drop in the level of G6PDH activity in this phase is also observed in figure 3.2-f. In relation to biomass and total protein concentration, MDH was the only enzyme that increased in activity level (Figure 3.2, g-j).

**Phase 7**
Phase seven is at 41 hours in SBD4. This phase represents the last peak in the CO₂ data and the phase was determined by the depletion of all the carbon sources in the medium. At 42 hours the last off-line sample analyses indicated a lower biomass concentration than phase 7. MDH and ADH levels were higher after phase 7. Hexokinase level had stabilised the and G6PDH activity level was declining.

Intracellular enzyme production in relation to biomass formation is described in section 3.1.3. The analysis is used to optimise the harvest time of the product enzymes.
Figure 3.2-a CO₂ exit gas profile - - - and DOT profile - - - of single batch culture 'SBD4' on defined medium. Six phases specified here are those reported by Locher et al. (1993, a). Phase 1 and phase 2 express the exponential growth of biomass on glucose. Glucose is utilised in phase 1, ethanol, glycerol, and organic acids (such as pyruvic acid, acetic acid, and propionic acid) are being produced. The cells are rich in carbon sources and short in energy. Phase 2 is when the growth on glucose changes characteristics. Phase 3 characterises the consumption of pyruvic acid and phase 4 is the consumption of acetic acid and pyruvic acid. The metabolic background for phase 5 was reported to be unknown and phase 6 is the utilisation of ethanol. Phase 7 at 41 hours, is not shown in this figure, is determined by total utilisation of all carbon sources.

Figure 3.2-b Off-line data of glucose - ● -, ethanol - ▲ -, and pyruvate - ○ - profile in single batch SBD4 with defined medium. Glucose and pyruvate are fully consumed at 25.5 hours where ethanol is at its maximum after the first CO₂ peak has occurred. Ethanol is fully utilised at 40 hours.
Figure 3.2-c  Dry cell weight concentration - □ - and total protein content - ■ - analysis of single batch fermentation SBD4 in defined medium. Dry cell weight increases up to 4.17 g/L in phase 2. It then starts its slow growth phase in phase 3 through to phase 4 of the CO₂ exit gas, where the second exponential growth phase of biomass occurs. At the end of phase 6 the ethanol is depleted and the biomass stops its growth. Total protein content of the sample has a value of 3.9 mg protein/mL of the sample.

Figure 3.2-d  Dry cell weight concentration - □ - and total protein content - ■ - analysis of single batch fermentation SBD4 in defined medium. Dry cell weight increases up to 4.17 g/L in phase 2. It then start its slow growth phase in phase 3 through to phase 4 of the CO₂ exit gas, where the second exponential growth phase of biomass occurs. At the end of phase 6 the ethanol is depleted and the biomass stops its growth. Total protein content of the sample has a value of 0.7 mg protein/mg biomass.
Figure 3.2-e  Off-line assay of Alcohol dehydrogenase - \( \triangle \) - (U/mL) and Malate dehydrogenase - \( \triangledown \) - (U/mL) in single batch fermentation. There is a rapid rise of ADH as glucose is limited. The activity of ADH appears to reach its maximum level at the end of phase 2 after the first CO\(_2\) peak when MDH starts its activity. MDH activity increased during the transition growth on ethanol.

Figure 3.2-f  Glucose-6-phosphate dehydrogenase activity levels U/mL - \( \triangledown \) - and Hexokinase activity levels U/mL - \( \circ \) - in single batch fermentation SBD4. G6PDH enzyme activity level increases in phase 1 through to phase 3 where the activity of this enzyme stabilises at the end of phases 3 to the middle of phase 4 as the biomass is growing on ethanol. It finally starts declining from phase 6. In phase 7 the activity is reaching zero. Hexokinase enzyme activity is following the same profile as biomass.
Figure 3.2-g Off-line assay of Alcohol dehydrogenase - △ - and Malate dehydrogenase - ▼ - in single batch fermentation. A rapid rise of ADH as glucose is limited. The activity of ADH appears to reach its maximum level in phase 2 at the first CO₂ peak where MDH starts its activity. MDH activity increased during the transition growth on ethanol.

Figure 3.2-h Glucose-6-phosphate dehydrogenase activity levels U/gDCW - ▼ - and Hexokinase activity levels U/gDCW - ♦ - in single batch fermentation SBD4. G6PDH enzyme activity level increases in phase 1 through to phase 3. The activity of this enzyme stabilised in phase 4. It finally starts decreasing at phase 6. After phase 7 the activity is reaching zero. Hexokinase enzyme activity level has a minimum value in phase 1. This enzyme activity level immediately reaches its maximum level and slowly decreases throughout the rest of cultivation time.
Figure 3.2-i Off-line assay of Alcohol dehydrogenase - △ - and Malate dehydrogenase - ▼ - (U/mg protein) in single batch fermentation. The activity of ADH appears to reach its maximum level in phase 2 at the first CO₂ peak where MDH starts its activity. MDH activity increased during the transition growth on ethanol.

Figure 3.2-j Glucose-6-phosphate dehydrogenase activity levels U/mg protein - ▼ - and Hexokinase activity levels U/mg protein - ♦ - in single batch fermentation SBD4. G6PDH enzyme activity level increases in phase 1 through to phase 5. It finally starts decreasing at phase 6. After phase 7 the activity is reaching zero. Hexokinase enzyme activity level has a minimum value in phase 1. This enzyme activity level immediately reaches its maximum level and stabilizes throughout phases 4 to 7.
3.1.3 Biomass formation and intracellular enzyme products

In the previous section correlations of some off-line fermentation measurement profiles with the CO₂ and DOT profiles were investigated for the single batch SBD4. The correlation of biomass formation with intracellular enzyme production in SBD4 is discussed in this section.

The diauxic growth pattern of *S. cerevisiae* in an aerobic batch culture (Sonnleitner and Kappeli, 1986) is widely accepted. Under conditions of aerobic ethanol formation, with glucose as the sole carbon source, glucose degradation takes place via two pathways. One pathway yields biomass and CO₂ (oxidative metabolism) and the second pathway produces ethanol and CO₂ (reductive metabolism) due to the conversion of pyruvate to acetaldehyde and reduction to ethanol. The combination of these two metabolisms is called oxidoreductive metabolism. The biomass growth is divided into two growth phases as shown in figure 3.2-c. In the first growth phase biomass is consuming glucose while ethanol is accumulated. After a lag phase, during which glucose is totally exhausted, the second growth phase is started where ethanol is the main carbon source. In this section formation of intracellular products are described in relation to biomass formation.

Figures 3.3,a-d indicate that biomass concentrations of up to 4 g/L represent the first growth phase. From 4 g/L to 4.5 g/L biomass the lag phase or the slow growth phase is shown and concentrations higher than 4.5 represent the second biomass growth phase. Logarithmic plots of intracellular enzymes and biomass formation (Figures 3.3,a-d) show the intracellular enzyme activity measurements of SBD4 where the samples were taken from the early exponential biomass growth phase to the end of growth on ethanol (Figure 3.2-c). The solid line on the graphs represent the unit slope and the dashed line is the best linear fit through the points.

**ADH**

The log-log axis of figure 3.3-a show that different proportional relationships hold over the two biomass growth phases. Visual comparison between the two slopes of the dashed lines and the unit slope line, indicates a higher rate of ADH production in the first growth phase than the second growth phase in SBD4.
Due to the limited respiratory capacity in the first growth phase when glucose flow exceeds the respiratory capacity, the residual part of glucose that can not be pass the bottleneck is metabolised reductively and ethanol is formed which is supplied to the medium. Hence the biomass produces the necessary ADH to enhance the reductive process. Glucose was finally exhausted at biomass concentrations of 4 g/L, and biomass was then adapting to the new environmental conditions where ethanol was going to be the main carbon source. As the cells started using ethanol, ADH was produced for the formation of acetaldehyde and further metabolism (Figure 1.3).

Four ADH isozymes have been established as being present in S. cerevisiae (Mauricio and Ortega, 1993). ADH-I, the fermentative form transforms acetaldehyde into ethanol. ADH-II, the oxidative form, oxidises the ethanol formed during the fermentation to acetaldehyde, which is then metabolised via the TCA cycle. ADH-III, occurs in the mitochondria and ADH-IV is in the cytoplasmic form. Mauricio and Ortega, (1993) studied the influence of fermentation conditions (strict anaerobic, semi-aerobic and short aeration) of grape musts on the specific activity of isozymes ADH-I and ADH-II. ADH-I was reported to be synthesised during the lifetime of the early generation of the fermentation, whereas ADH-II behaved as a constitutive enzyme under all fermentation conditions. Fowler (1972) reported the presence of ADH-I under all growth conditions and the presence of ADH-II only under conditions which favoured oxidative metabolism. Figure 3.3-a shows the total ADH activity level (U/mL) in the cells. Hence, cells living on glucose are more efficient in producing ADH than those growing on ethanol.

Hexokinase

Hexokinase activity (U/mL) production of SBD4 in relation to biomass formation is illustrated in figure 3.3-b. There was a linear relationship between the enzyme and biomass production over the whole range of biomass concentration. Hexokinase is a constitutive enzyme the enzyme being present in constant amount regardless of the metabolic state of the cell. At biomass concentrations of 1.8 g/L hexokinase is inhibited (as described in section 3.1.2, phase 1). The slope of the dashed line in figure 3.3-b is parallel to the unit slope indicating the same rate of enzyme production as that of biomass formation. Boucher (1985) reported the synthesis of this enzyme throughout the stationary phase of S.cerevisiae. This observation is also indicated in figure 3.3-b where the enzyme was synthesised at very slow rate.
MDH

Figure 3.3-c shows the changes in MDH activity levels of SBD4 as biomass was formed in the first and second growth phase. Although the activity of this enzyme was very low (0.03-0.3 U/mL) in the first biomass growth phase, formation of the enzyme was directly proportional to the biomass formation. At biomass concentration of 4 g/L when the glucose was totally exhausted and the cells had started the slow growth phase (lag phase), the enzyme activity level showed a significant increase.

MDH is an induced enzyme and the sudden change in the activity level of this enzyme can be explained by the presence of ethanol as the only carbon source. As the inducable enzyme MDH is being made only when the enzyme is needed. The cells began to synthesise MDH as soon as the glucose was exhausted.

MDH activity in the second growth phase increased at the same rate as the biomass formation. The two dashed lines in figure 3.3-c are parallel to the unit slope line. Efficiency of production of this enzyme by the biomass was much higher when the cells growing on ethanol. Polakis and Bartly (1965) also reported an increase in the activity level of MDH from 450 U/mg protein in the first exponential growth phase to 3060 U/mg protein in the second exponential growth phase. A sudden increase in the activity level of isocitrate lyase was also reported (Polakis and Bartly, 1965). Zeng and Deckwer (1994) reported the repression of TCA cycle activity but not the respiratory capacity in the presence of glucose. Figure 3.3-c, however, shows the formation of MDH enzyme at low levels. Therefore as the enzyme was being synthesised at very low levels the TCA cycle was active at a very slow rate.

G6PDH

G6PDH is constitutively produced as G6PDH is directly proportional to the biomass formation for biomass concentrations of up to 6 g/L (Figure 3.3-d). This range of concentration was through the first exponential growth phase to the middle of the exponential biomass growth phase (Figure 3.2-c). The linear relationship indicates that the rate of biomass formation was the same as the enzyme production rate. Degradation of this enzyme occurred at biomass concentration of about 6 g/L, when the cells lost efficiency in production of G6PDH and ethanol was at very low concentration. This state was used for further decision making on the harvest time at this stage of the work.
Figures 3.3,a-d indicate that at biomass concentrations of 4-4.5 g/L (lag or slow growth phase) the rate of change of enzyme production was linear in the case of G6PDH (3.3-d) and hexokinase (3.3-b) whereas there was hardly any noticeable for ADH (3.3-a) and presents the maximum change for MDH production (3.3-c) was between the two growth phases of biomass production.

Comparison of the CO$_2$ exit gas profile with the off-line sample analysis identified the correlations of the intracellular enzyme products with different phases of the CO$_2$ pattern (section 3.1.2). Investigating the reproducibility of the correlations between on-line variables and off-line measurements, meant that a decision could be made on the timing of product yield and automation of the process.

The reproducibility of correlations between on-line and off-line variables of SBD4 was investigated from phase 1 to phase 4 of the fermentations on the defined medium, by repeating the experiment under the same fermentation conditions. Original results of these experiments are illustrated in appendix 2.1, 2.2, and 2.3. For ease of visual comparison of these profiles, components of different batches are plotted on the same diagram and based on the CO$_2$ exit gas analysis data in section 3.1.2. The first batch of the repeated batch culture RBD2-1 is also be included as single batches, since this experiment was carried out under same fermentation conditions.
Figure 3.3-a The log-log plot of ADH product formation in relation to biomass concentration. Data are from fermentation SBD4. Plot shows the different proportional relationships for the two biomass growth phases. The solid line on the graph represent the unit slope through the point $(1,1)$ so that the rates can be compared.

Figure 3.3-b The log-log plot of hexokinase product and biomass formation in SBD4. A proportional relationship holds over the two biomass growth phases. The plot also shows the inhibition of this enzyme at 1.8 g/L biomass concentration. The solid line on the graph represents the unit slope through the point $(1,1)$ so that the rates can be compared.
Figure 3.3-c  The log-log plot of MDH production and biomass formation during fermentation SBD4. Plot shows different proportional relationships over the first and second biomass growth phases. A sudden change in the activity level for this product is shown at the end of first growth phase when glucose is depleted and TCA cycle is activated. The solid line on the graph represent the unit slope through the point (1,1) so that the rates can be compared.

Figure 3.3-d  The log-log plot of G6PDH product formation in relation to biomass concentration in SBD4 fermentation. A proportional relationship holds for biomass concentrations of up to 6 g/L when the plot shows degradation of this product. The solid line on the graph represent the unit slope through the point (1,1) so that the rates can be compared.
3.1.4 Reproducibility of the off-line analysis profiles in batch fermentations with defined medium

In section 3.1.2 correlations of some off-line fermentation measurement profiles with CO₂ and DOT profiles were investigated in single batch cultivations with defined medium. SBD4 was chosen as an example to describe the existing correlations and profiles of on-line and off-line measurements at different fermentation phases. The reproducibility of these profiles is studied in this section.

<table>
<thead>
<tr>
<th>Batch Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBD1</td>
</tr>
<tr>
<td>SBD2</td>
</tr>
<tr>
<td>SBD3</td>
</tr>
<tr>
<td>SBD4</td>
</tr>
<tr>
<td>RBD2-1</td>
</tr>
<tr>
<td>RBD2-1</td>
</tr>
<tr>
<td>-------</td>
</tr>
</tbody>
</table>

Table 3.2 A matrix of fermentation experiments using defined medium. Emboldened experiments are included in this section.

A matrix of the fermentation data is shown in Table 3.2. The emboldened experiments are those included in this section. Phases 1-4 of these fermentations are presented in comparison graphs (Figures 3.4,a-r). RBD2-1 is also considered as a single batch fermentation in this section. RBD2-1 is the first batch of a repeated batch fermentation, RBD2, which was also inoculated from a shake flask and the same fermentation technique and conditions were followed as in the SBD fermentations. The initial working volume for SBD4 was 5.5 litres whereas SBD1, SBD2, SBD3, and RBD2-1 had an initial working volume of 5 L. RBD1-1 is not included in this section since fermentation data for this experiment are only available for phases 3 to 7 whereas the comparison of single batch cultures is from phase 1 to phase 4 of the CO₂ exit gas analysis.
To compare results from different batches relative to the CO$_2$ peak, the fermentation time axis had to be transformed. Fermentation time was expressed relative to the first CO$_2$ peak by subtracting the time at which the peak occurred from the actual fermentation time.

Relative fermentation time for each experiment was calculated with reference to the time of the first CO$_2$ peak from the mass spectrometer data. As detailed in chapter 2, CO$_2$ data were recorded at three minutes intervals for about forty hours (which corresponds to approximately 550 on-line data). Due to the large number of data points, the function 'MAX(A1:A2)' within Microsoft Excel (chapter 2) spreadsheet was used to identify the time at which the first CO$_2$ peak (maximum CO$_2$ data point) occurred. This time was taken as the reference time and subtracted from the fermentation time to give the relative fermentation time (see equation 3.1).

$$T_{\text{rel}} = T_{\text{ferm}} - T_{\text{cmax}}$$ (3.1)

where $T_{\text{ferm}}$ is the actual fermentation time from inoculation, $T_{\text{cmax}}$ is the time at which maximum CO$_2$ occurs and $T_{\text{rel}}$ is the relative fermentation time based on the first CO$_2$ peak.

Extracellular and intracellular components from different batches were then plotted on the same graph against the relative fermentation time. This plot was used to investigate the reproducibility of the profiles for later process decision making.

Figures 3.4,a-r illustrate profiles of the same components from different single batch experiments SBD1, SBD2, SBD3, SBD4, and RBD2-1. Logarithmic plots are used to linearise the profiles before and after the first CO$_2$ peak. Joining the data points with dashed lines on the graphs does not help clarifying the overall picture since each graph contains information from five different fermentations. Hence, the points on the graphs are not joined with dashed lines. In Figure 3.4,a-r, the relative fermentation time zero is when the CO$_2$ exit gas concentration has reached the maximum value, which is the first CO$_2$ peak, that also indicates phase 2 of the CO$_2$ profile. The time length for phase 2 was around two hours for the single batch fermentations presented in section 3.1.1. Phase 2 for these fermentations was considered to be ±1 hour of time zero on the x-axis if these plots.
**Glucose consumption**

Glucose consumption profiles (g/L) are illustrated in Figure 3.4-a. Glucose was exhausted in phase 2 of the CO$_2$ profile. From variability analysis studies (Chapter 4, section 4.3.6) for glucose concentrations of up to 17 g/L there was a standard deviation of ±0.3% and for samples with higher glucose concentration a standard deviation value of ±0.053 g/L was expected. Only three points are observed for SBD2 (- o -) in Figure 3.4-a, indicating the three samples taken before the relative time zero. From the original graphs (Appendix 2.2) the fourth sample showed a zero concentration of glucose a few minutes after the first CO$_2$ peak had occurred. Therefore later samples are not shown on the log plot of the glucose comparison graph.

**Ethanol production**

Ethanol reached a maximum value in phase two of the CO$_2$ exit gas analysis (Figure 3.4-b). The maximum concentration of ethanol production shows an accumulation of up to 10 g/L ethanol at relative time zero. Although the same samples as those for glucose were used for ethanol assays in the HPLC analysis, data variation were more noticeable for ethanol due to the sensitivity of this component. Variability of the ethanol assay was probably due to freezing the samples and evaporation of ethanol when the samples were being defrosted for HPLC analysis.

**Biomass production**

Biomass production rate was obtained from two sets of measurements. Dry cell weight concentration and optical density measurements (Figures 3.4-c, 3.4-d). In Figure 3.4-c, the dry cell weight concentration for SBD1 shows a higher level for the profile of this variable than the other batches presented in this graph. For all batches dry cell weight concentration increased exponentially until relative time zero when the concentration stabilised. All batches had the same rate of production of biomass and the same final biomass concentration of 4 ± 0.3 g/L (Figure 3.4-c). SBD2 shows some deviation on the first two OD measurements. For the first two OD values of SBD2, the spectrophotometer calibration was incorrect. The slow growth phase of the biomass after relative time zero shows less variability than that of dry cell weight concentration graphs. Reproducibility of the data in Figures 3.4-b and 3.4-c indicates that the first CO$_2$ peak in the exit gas analysis is an indication of the biomass transition phase.
**Total protein concentration**

Total protein concentration also followed the same profile as the biomass concentration. From Figure 3.4-e, the protein concentration (mg/mL) stabilised in the time range from half an hour before (SBD4) to half an hour after the CO\(_2\) peak (SBD1-3 and RBD2-1) indicated by relative fermentation time zero on the x-axis of Figure 3.4-e. Specific protein concentration of the samples before and after the CO\(_2\) peak are illustrated in Figure 3.4-f. Batch to batch variability observed in specific protein concentration before CO\(_2\) peak was greater than that after the peak (zero hour at relative time axis). SBD4 has a different protein concentration level at phase 1 than the rest of the batches. The larger initial working volume for this batch is a possible explanation for this observation. SBD4 was started at 5.5L since more samples were required for analysis of the broth at all phases in this batch. Specific protein concentration values represents the amount of protein accumulated inside the cell. The difference in the specific activity level of the fermentations before and after relative time zero is considered yo be due to an accumulation of protein inside the cell caused by a sudden increase in MDH and other TCA cycle enzymes at this phase.

**Intracellular enzyme activity**

Intracellular enzyme content of the cells are also compared in this section, the profiles of the enzymes showed good reproducibility but the enzyme levels present some variations. Enzyme activities are shown in Figures 3.4g-r. The plots present the activity of each enzyme in terms of U/mL, U/mg biomass, and U/mg protein. Although in general the profiles follow a consistent trend, a large variability is observed in U/mg protein graphs. Enzyme activity in U/ml of the broth shows less variability.

**ADH**

The profiles of ADH enzyme activity in single batch defined fermentations are illustrated in Figures 3.2-g (U/mL), 3.2-h (U/mg biomass), and. 3.2-i (U/mg protein). An initial rapid rise in ADH activity (U/mL) in phase 1 and phase 2 of CO\(_2\) exit gas analysis was followed by stabilisation of the enzyme activity from relative time zero when glucose was completely utilised. ADH activity (U/mg) shows the same profile indicating an increase in the enzyme activity per mg of biomass before the relative time zero. Variation of data points on U/mg (Figure 3.4-h) biomass graph is significantly higher than variations from the U/mL graph (Figure 3.4-g). Activity of this enzyme however stayed constant through the phases concerned here in accordance with the total protein concentration (Figure 3.4-g). Better consistency of ADH activity profiles in U/mg biomass was observed (Figure 3.4-h) than that in U/mg protein graphs (Figure 3.4-i).
**G6PDH**

G6PDH activity levels are presented in Figures 3.4-i,j,k. An initial slow rise in the enzyme activity is followed by stabilisation of this enzyme after relative time zero. Increase in the activity at phase 1 and phase 2 is also noticed in the unit activity of the enzyme per mg of dry cell weight. Activity levels per mg of protein almost stayed the same across the transition phase (phase 2). A much higher activity level for this enzyme was observed in SBD1. A PU8800 UV/VIS was used to measure the activity of G6PDH for SBD1. The spectrophotometer was not temperature controlled since the thermocirculator to absorbance cell was not working. There was a noticeable temperature difference at the end of the analysis, the temperature had increased during the day. Using a different instrument for measurement of this enzyme and the change in temperature has affected the profile of G6PDH in SBD1. Consistency of data in G6PDH activity measurements are significantly greater than the data consistency in ADH activity measurements. Figure 3.4-1 shows similar activity of the enzyme before and after the CO₂ peak for the single batch fermentations presented in this section.

**MDH**

The MDH activity level in single batch fermentations are indicated in Figures 3.4-m, n, o. MDH assay did not show any activity for SBD3 and RBD2-1. The profile of this enzyme for SBD1 followed the same profile as that of the biomass. This was only observed for SBD1. Later analysis of the enzyme for repeated batch cultures indicated the reproducibility of the profile as observed for SBD2 and SBD4. The MDH assay procedure was different for SBD3, RBD2-1, and RBD2-2 than that reported in section 2.3.4. The difference was in the sample size and the amount of cofactor used for the assay. Sample size was twice that reported in section 2.3.4 and the cofactor was half the amount stated in the MDH assay procedure. Therefore the reaction would have taken place before any activity could be detected. The activity of this enzyme before the first CO₂ peak at relative time zero is about 0.1 U/mL. The activity of the MDH started in the middle of phase 2, about relative time zero. There was a sudden rise from 0.1 U/mL to 2 U/mL at relative time zero for SBD2 and SBD4 in Figure 3.4-m. This sudden change in the activity was also noticed in unit activity per gram biomass graphs (Figure 3.4-n) and unit activity per mg protein (Figure 3.4-o). MDH, the most active enzyme of the TCA cycle was repressed while the biomass was consuming glucose and started being active as soon as the glucose was exhausted. This transition was clearly observed in the SBD4 and SBD2 fermentations.
Hexokinase

Hexokinase was measured for SBD3, SBD4, and RBD2-1. Figures 3.4-p,q,r indicate an increase in the activity of the enzyme throughout phase 1. Hexokinase activity stabilised after phase 2 of the CO₂ exit gas profile (Figures 3.4-p). This enzyme is growth linked and the enzyme level increased as the biomass was growing. The activity of this enzyme has stayed constant throughout the phases 1-4 (Figures 3.4-q,r). The two points at about -2 and 2 hours represent the inhibition of this enzyme which can be caused by high levels of ATP, H⁺, citrate, and early intermediates of the TCA cycle as explained in section 3.1.2, phase 1. Enzyme activity profile for hexokinase is highly consistent in figures 3.4-p,q,r.

In this section the relationships between CO₂ exit gas analysis phases and off-line sample measurements of single batch fermentations on defined medium are discussed. Phase 2 and phase 3 of the profile indicated depletion of glucose, maximum formation of ethanol, termination of the first exponential growth phase of the biomass, and the maximum changes in the MDH intracellular enzyme activity. Phases 1-4 were therefore identified as phases of interest. Reproducibility of on-line and off-line correlations for these phases in single batch fermentations with defined medium were investigated by repeating the single batch fermentations under the same experimental conditions.

Good reproducibility of the relationships based on the first CO₂ peak was observed in phases 1-4. However, the level of the profiles varied for individual batches indicating the process variability of single batch fermentations. Variations in the profile levels could be due to inoculum age difference (for SBD2 and SBD4, the fermenter was not ready to be inoculated after 72 hours, the inoculum was kept in the cold room for an extra 24 hours), assay mixture preparation, and sterilisation time of the medium. Although assay mixture preparation and sterilisation time of the medium were kept constant, these factors could not always be exactly the same.

To reduce the batch to batch variability observed in this section and to eliminate the inoculum age effect, a series of repeated batch cultures were carried out. The reproducibility of the different batches within the repeated batch cultures are discussed in section 3.2.
Figure 3.4-a  Off-line data of glucose consumption (g/L) profile from single batches with defined medium SBD1 - □ -, SBD2 - o -, SBD3 - ▲ -, SBD4 - • -, and RBD2-1 - v -. 

Figure 3.4-b  Ethanol production profile (g/L) from single batches with defined medium SBD1 - □ -, SBD2 - o -, SBD3 - ▲ -, SBD4 - • -, and RBD2-1 - v -. 

SBD 'comparison graphs'
Figure 3.4-c  Dry cell weight concentration (g/L) profiles from single batches with defined medium SBD1 - □ - , SBD2 - ○ - , SBD3 - △ - , SBD4 - ♦ - , and RBD2-1 - V -.

Figure 3.4-d  Optical density measurements of biomass concentration. Profiles in single batches with defined medium SBD1 - □ - , SBD2 - ○ - , SBD3 - △ - , SBD4 - ♦ - , and RBD2-1 - V -.
Figure 3.4-e  Total protein concentration profiles in single batches with defined medium SBD1 - □ -, SBD2 - ○ -, SBD3 - ▲ -, SBD4 - ● -, and RBD2-1 - ▼ -.

Figure 3.4-f  Total protein concentration profiles in single batches with defined medium SBD1 - □ -, SBD2 - ○ -, SBD3 - ▲ -, SBD4 - ● -, and RBD2-1 - ▼ -.
Figure 3.4-g  ADH enzyme activity (U/mL) profiles from single batches with defined medium SBD1 - □ - , SBD2 - ○ - , SBD3 - ▲ - , SBD4 - ● - , and RBD2-1 - ∨ - .

Figure 3.4-h  ADH enzyme activity (U/g DCW) profiles from single batches with defined medium SBD1 - □ - , SBD2 - ○ - , SBD3 - ▲ - , SBD4 - ● - , and RBD2-1 - ∨ - .
Figure 3.4-i  ADH enzyme activity (U/mg protein) profiles from single batches with defined medium SBD1 - □ -, SBD2 - ○ -, SBD3 - ▲ -, SBD4 - ♦ -, and RBD2-1 - ▽ -. 

Figure 3.4-j  G6PDH enzyme activity (U/mL) profiles from single batches with defined medium SBD1 - □ -, SBD2 - ○ -, SBD3 - ▲ -, SBD4 - ♦ -, and RBD2-1 - ▽ -.
Figure 3.4-k  G6PDH enzyme activity (U/g DCW) profiles from single batches with defined medium SBD1 - □ -, SBD2 - ○ -, SBD3 - ▲ -, SBD4 - ● -, and RBD2-1 - ▼ -.

Figure 3.4-l  G6PDH enzyme activity (U/mg protein) profiles from single batches with defined medium SBD1 - □ -, SBD2 - ○ -, SBD3 - ▲ -, SBD4 - ● -, and RBD2-1 - ▼ -.
Figure 3.4-m MDH enzyme activity (U/mL) profiles from single batches with defined medium SBD1 - □ -, SBD2 - ◦ -, SBD3 - ▲ -, SBD4 - ♦ -, and RBD2-1 - V -. 

Figure 3.4-n MDH enzyme activity (U/g DCW) profiles from single batches with defined medium SBD1 - □ -, SBD2 - ◦ -, SBD3 - ▲ -, SBD4 - ♦ -, and RBD2-1 - V -.
Figure 3.4-o MDH enzyme activity (U/mg protein) profiles from single batches with defined medium SBD1 - □ -, SBD2 - ○ -, SBD3 - ▲ -, SBD4 - ♦ -, and RBD2-1 - ▼ -.

Figure 3.4-p Hexokinase enzyme activity (U/mL) profiles from single batches with defined medium SBD3 - ▲ -, SBD4 - ♦ -, and RBD2-1 - ▼ -.
Figure 3.4-q  Hexokinase enzyme activity (U/g DCW) profiles from single batches with defined medium SBD3 - ▲ - , SBD4 - ● - , and RBD2-1 - ▼ -.

Figure 3.4-r  Hexokinase enzyme activity (U/mg protein) profiles from single batches with defined medium SBD3 - ▲ - , SBD4 - ● - , and RBD2-1 - ▼ -.
3.2 Repeated batch cultures.

The reproducibility in the levels of some intracellular and extracellular components from phases 1 to 4 of CO₂ exit gas analysis profile of single batch fermentations were investigated in section 3.1.4. For all components under investigation profiles showed a reasonable degree of reproducibility. Enzyme activity levels and concentration levels varied for different batches. Variations were assumed to have come from: the sterilisation procedure, the assay mixture preparation, diluting the samples for off-line analysis, and the inoculum age difference between single batch fermentations (inoculum for SBD2 and SBD4 was 24 hours older than inoculum for SBD1 and SBD3).

To reduce the batch to batch variability observed in the single batch fermentations, experiments were designed as repeated batch cultures with the same fermentation conditions as the single batch fermentations. The experimental results of repeated batch cultures are presented in appendices 3, 4, and 5. Analysis of the repeated batch culture results are shown in this section to compare the reproducibility of the on-line and the off-line variables of these cultures with the single batch fermentations (Section 3.1.4).

To develop the repeated batch technique, preliminary fermentations were designed and carried out as aerobic yeast repeated batch cultures of three to four batches per run at a 5L scale in chemically defined medium. Section 3.2.1 presents an example of these fermentations to investigate the reproducibility of the CO₂ and DOT profiles of repeated batch fermentations.

To reduce the observed batch to batch variability in single batch experiments, repeated batch cultures, RBD1, RBD2 and RBD3, were carried out as emboldened in Table 3.3. Inoculum for the first batch was 250 mL from a shake flask prepared 72 hours prior to fermenter inoculation. Consecutive batches followed by self inoculating the fermenter (Chapter 2, section 2.1.6). Off-line samples were measured during the exponential growth phase through the transition into the stationary phase of the microbial growth which is referred to as phase 1 to phase 4 of the CO₂ exit gas analysis profile. Experimental results (Appendices 3, 4, and 5) show on-line data corresponding to fermentation time of the off-line analysis.
### Table 3.3

Experiments discussed in this section are emboldened according to the type of fermentation. SBD is the single batch fermentation with define medium. RBD is the repeated batch fermentation with define medium.

<table>
<thead>
<tr>
<th>Batch Identification</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SBD1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBD2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBD3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBD4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBD1-1</td>
<td>RBD1-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBD2-1</td>
<td>RBD2-2</td>
<td>RBD2-3</td>
<td></td>
</tr>
<tr>
<td>RBD2-2</td>
<td>RBD2-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBD2-3</td>
<td>RBD3-2</td>
<td>RBD3-3</td>
<td>RBD3-4</td>
</tr>
</tbody>
</table>

Individual batches from RBD2 and RBD3 are compared within the repeated batch culture sets. Results are analysed using the same technique as the single batch comparison analysis. DOT data were used in RBD2 fermentation and CO₂ data were used in RBD3 fermentation to calculate the relative fermentation times. Comparison of the single batch cultures within a repeated batch culture was then based on the calculated relative times. Comparison of results from repeated batch fermentations to the single batch and the reproducibility of the repeated batch data is discussed in sections 3.2.2 and 3.2.3.
3.2.1 Preliminary repeated batch experiments with defined medium

Preliminary repeated batch cultures (PRB1, PRB2, and PRB3) provided a series of data files from the on-line variables of these fermentation. A high degree of reproducibility was observed from CO₂ exit gas profiles and the DOT profiles of these repeated batch cultures within each experiment.

CO₂ exit gas analysis and DOT data were recorded at three minutes intervals from a mass spectrometer and a DOT probe. Figures 3.5-a and 3.5-b show a typical CO₂ and DOT profile from repeated batch experiment PRB1 as an example of the preliminary experiments carried out. PRB2 and PRB3 showed the same on-line profiles as PRB1. The fourth batch shows on-line data from refill to the end of the last fermentation phase (phase 7). The repeatability of DOT profiles indicated that DOT profiles can also be used to recognise the transition phase. The first trough from the DOT profile happens 0.15 to 0.2 hours after the first CO₂ exit gas peak (section 3.2.2, RBD2). This parameter can be used for further process decision making where mass spectrometer data are not available.

The inoculum for the first batch was prepared on the same chemically the defined medium as the medium for the fermentation. For the subsequent batches the fermenter was self inoculated during the refill procedure (chapter 2, section 2.1.6.1). The refill procedure was performed manually at phase 4 of each batch. The refill process could either be based on time or a specific phase of the fermentation. For PRB1, refill (or inoculation) was at phase 4 of the CO₂ profile for all batches (Figures 3.5-a,b).

A difference in the amplitude of the CO₂ exit gas profile was observed for consecutive batch cultivations of PRB1 (figure 3.5-a). This variation was established to be due to the inaccuracy of inoculum size left in the fermenter, since the inoculum was 250 mL of the previous batch left in the fermenter. Refill was carried out manually, using the volume calibration scale on the fermenter which was for the total working volume of 5L ± 50 mL. Therefore accuracy of inoculum size in the refill process was not as good as the accuracy of other fermentation conditions.

Nevertheless, repeated batch cultures with defined medium were carried out as this technique gave the best means of standardising a batch fermenter and being comparable with an industrial process. Broth samples of individual batches were analysed from phase 1 to phase 4 of the CO₂ exit gas profile (Section 3.2.2).
Figure 3.5-a  A graphical presentation of CO₂ exit gas profile in repeated batch culture (PRB1) on defined medium.

Figure 3.5-b  DOT profile of the same experiment (PRB1) is presented. Data were obtained from the in situ probe at three minute intervals. Good reproducibility is observed as with the CO₂ exit gas profiles.
3.2.2 Repeated batch fermentation RBD1

RBD1 consisted of two batches. First batch RBD1-1, was inoculated from a 250 mL shake flask. However the data acquisition system had failed overnight during the first batch. After reinitialising the Real Time Data Acquisition System, the first off-line sample from RBD1-1 was analysed. There was no indication of the CO\(_2\) exit gas phase at the timing of the first off-line sample. About two hours after reinitialisation of the data monitoring system, the second peak of the CO\(_2\) profile was observed indicating that off-line samples were taken from the beginning of phase 3 (See appendix 3.1, Figure A3.1-a). Data from this batch were used to confirm the SBD4 results in phases 4 to 6 (section 3.1.2). RBD1-2 was the second batch of RBD1 fermentation. Inoculum for RBD1-2 was 1 litre of the remaining cells from RBD1-1. RBD1-2 was sampled from phase 1 to phase 4 of the CO\(_2\) exit gas profile. Fermentation results of RBD1 are included in appendix 3. The two batches of RBD1 were analysed for different phases of CO\(_2\) exit gas profile. Comparison of individual batches of RBD1 is not included in this section due to the inconsistent data from this fermentation. Analysis of RBD2 and RBD3 are discussed in the next two sections.

3.2.3 Analysis of repeated batch fermentation RBD2

This fermentation was a repeated batch experiment with three batches RBD2-1, RBD2-2, and RBD2-3 (Appendix 4.1, 4.2, and 4.3). Due to mass spectrometer failure during experiment RBD2-2, exit gas analysis data were not available for this experiment. However, on-line DOT data (Figure 3.6) were used to estimate the time of the first CO\(_2\) peak from exit gas analysis.

From on-line data of previous fermentations (Appendices 2, 3, 5) a time difference of 0.15 to 0.2 hours between the CO\(_2\) peak and the DOT trough can be observed. This difference was used to estimate the timing of the CO\(_2\) peak in RBD2 (Table 3.5). Estimated CO\(_2\) peak time for RBD2-1 was in agreement with time noted from the mass spectrometer before the instrument had failed. The CO\(_2\) gas analyser connected to the fermenter also showed the first peak at 67.3 h in RBD2-3. This procedure demonstrated that the available DOT profile could therefore be used to compare the off-line analysis of fermentations.
<table>
<thead>
<tr>
<th></th>
<th>Monitored DOT (h)</th>
<th>Estimated CO₂ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBD2-1</td>
<td>15.9</td>
<td>15.7</td>
</tr>
<tr>
<td>RBD2-2</td>
<td>46.15</td>
<td>46</td>
</tr>
<tr>
<td>RBD2-3</td>
<td>67.5</td>
<td>67.3</td>
</tr>
</tbody>
</table>

Table 3.4 Estimated fermentation time at which the first CO₂ peak had occurred for RBD2-1,3 from on-line monitored DOT data.

Relative fermentation times were then calculated (As described in section 3.1.4) based on the first CO₂ peak of the exit gas analysis. Individual components of each batch were then plotted on the same graph to investigate the reproducibility of the off-line analysis within a repeated batch culture (Figures 3.6, a-s).

RBD2-1 was inoculated from a 250 mL inoculum flask with inoculum age of 72 hours. Consecutive batches were started by refilling the fermenter (chapter 2, section 2.1.6). However, RBD2-2 was inoculated from the cells after phase 7 of RBD2-1 and RBD2-3 was inoculated in phase 6 of RBD2-2 (Figure 3.6).

![Figure 3.6 DOT profile of repeated batch culture on defined medium RBD2.](image)
Self inoculation of the fermenter was carried out manually, keeping the inoculum size at 250 mL. The number of sample points on the comparison graphs (Figures 3.6, a-s) indicate that RBD2-1 and RBD2-2 were sampled for a longer period of time before the relative time zero (first CO\textsubscript{2} peak) than RBD2-3. This difference in samples was due to the inoculum variations (manual refilling of the fermenter) and timing of the refill (refill at different CO\textsubscript{2} phases). The DOT profile of RBD2 is shown in Figure 3.6 from inoculation time of the fermenter (time zero). First off-line sample for RBD2-1 was taken 12 hours after inoculating the fermenter. For RBD2-2 and RBD2-3, samples were taken about half an hour to an hour after refilling the fermenter. Figure 3.6 indicates that there was a shorter time between the refill and first trough in RBD2-3 than that for RBD2-2. The most likely interpretation of this time length difference is the variation in inoculum size and/or self inoculating the fermenter at different phases indicating variation in inoculum age. A larger inoculum leads to a shorter time for substrate depletion and shorter time to reach the CO\textsubscript{2} peak. Inoculum to RBD2-2 was taken from biomass which had completely utilised ethanol whereas at the time of the second refill cells were still using ethanol and according to the DOT profile the yeast was in phase 6 of the CO\textsubscript{2} profile. However, further investigation is required to study the effect of self inoculating the fermenter at different CO\textsubscript{2} phases (Chapter 7, Recommendations).

Profiles of the same component from RBD2-1, RBD2-2, and RBD2-3 are illustrated in Figures 3.6, a-r. Logarithmic plots are used to linearise the data before and after the CO\textsubscript{2} peak. Relative fermentation time zero is when the maximum value of the CO\textsubscript{2} exit gas analysis from the on-line profile is obtained indicating the first CO\textsubscript{2} peak of the profile. Phase 2 of the profile is considered to be ±1 hour of the time zero on the x-axis. Similar y-axis scale is chosen for later visual comparison of the repeated batch fermentations to the single batch culture profiles.

**Glucose consumption**

The glucose consumption profile is shown in Figure 3.6-a. Glucose was exhausted in phase 2 of the profile (about an hour after the relative time zero). Figure 3.6-a indicates a better reproducibility in the level of the glucose consumption profile of RBD2 than single batch cultivations (Figure 3.4-a). The most likely explanation for this observation is that defined medium for the repeated batch culture was made in one batch therefore medium for RBD2-1, RBD2-2, and RBD2-3 has exactly the same composition and the medium had been sterilised under the same conditions. Whereas for single batch fermentations, although the same gradients of chemicals are used, the liquid medium was made in different batches at different days and sterilisation procedure could not be exactly the same. The variability in SBD cultures emphasises the importance of medium preparation and sterilisation in reducing the process variability of the fermentations.
**Ethanol production**

Ethanol production profile is indicated in Figure 3.6-b. Maximum concentration of ethanol has reached at relative time zero. An accumulation of up to 10 g/L of ethanol was observed for RBD2-1 and RBD2-2. In the case of RBD2-3 maximum ethanol concentration was of 8 g/L. A possible explanation for this variation is the history of the inoculum for this batch, where cells were from early in phase 6 of RBD2-2. In this phase, the biomass is using the remaining ethanol in the broth. An inoculum of this nature would be expected to use the produced ethanol more than cells which have been starved and then exposed to an environment of glucose as the sole carbon source, which was the case for RBD2-1 and RBD2-2.

**Biomass production**

Both dry cell weight and optical density measurements are presented (Figures 3.6-c,d) to study the biomass production in phases 1 to 4 of RBD2. Biomass stabilised after relative time zero when a maximum concentration of 4 g/L dry cell was obtained for individual batches of RBD2. Good reproducibility is observed for the profiles and biomass level after relative time zero. The outlier point in the optical density measurement for RBD2-1 at relative time zero is most likely to be due to processing of the off-line sample without mixing the broth sample taken from the fermenter before dilution for optical density measurements.

**Total protein concentration**

Closer protein concentration profiles were obtained from individual batches of RBD2 (Figures 3.6-e) than those observed in single batch fermentations (Figures 3.4-e,f) indicate a better reproducibility of total protein measurement and a smaller process variability for repeated batch cultures. The larger variability of specific protein concentration data (Figure 3.6-f), for before the relative time zero would be due to the small concentrations of protein and biomass analysis. As the biomass concentration increased, less variability was observed in the profile of the specific protein concentration.

**Intracellular enzyme activity**

Figures 3.6,g-r illustrate the intracellular enzyme activities in repeated batch culture RBD2. Activity of the enzymes are presented as U/mL, U/g biomass, and U/mg protein. Enzyme profiles of different batches follow a consistent trend. The variability observed in U/mL of the enzyme activity was less than that in U/g biomass and U/mg protein.
The profiles of ADH enzyme activity in RBD2 are illustrated in Figures 3.6-g (U/mL), 3.6-h (U/g biomass), and 3.6-i (U/mg protein). Although the maximum level of the profiles from RBD2 were similar to those from single batch fermentations. The closer profiles from individual batches of RBD2 indicate a better reproducibility for the ADH level in repeated batch cultures than in single batch fermentations.

G6PDH activity levels are presented in Figures 3.6-j, k, l. Similar activity levels and profiles are observed to those of single batch cultures. Figure 3.6-l indicates that specific activity of this enzyme in terms of protein concentration is almost constant throughout the phases presented (0.2-0.4 U/mg protein).

MDH activity level for RBD2 is indicated in Figures 3.6-m, n, o. Due to problems with the assay mixture, the enzyme analyses did not show any change in the activity of this enzyme for batches RBD2-1 and RBD2-2. The problem was detected and solved for RBD2-3 (section 3.1.4, MDH). Activity of this enzyme changed from 0.8 U/ml to 2 U/ml across the relative time zero.

Hexokinase activity levels are shown in Figures 3.6-p, q, r. Level of the profile (Figure 3.6-p, U/ml) is similar to those of single batch fermentations. Better reproducibility for the profile of this enzyme is shown for RBD2. Figures 3.6-p, q indicate that hexokinase activity was initially 800 U/mg biomass in phase 1 and reduced to 200 U/mg biomass at relative time zero then activity increased to 300 U/mg biomass and stabilised at relative time 1 hour. A similar activity profile is observed in terms of U/mg protein (Figure 3.6-r).

**Pyruvate production and consumption**

Figure 3.6-s illustrates production and consumption of pyruvate in repeated batch culture RBD2. A maximum pyruvate level of 0.18 g/L was observed at relative time zero for individual batches. A slower rate of production than the rate of consumption was indicated for all batches. Variability observed for the accumulation of pyruvate was considerably less than the variability for the rate of consumption of this component. Both ethanol and pyruvate reached their maximum concentration at relative time zero. A comparison of the profiles of these two compounds shows that the higher concentration of ethanol was reached at relative time zero when pyruvate had lower concentration for RBD2-1 (Figures 3.6-b and 3.6-s). Minimum production of ethanol and maximum pyruvate level at the same zero relative time was observed for RBD2-3.
Figure 3.6-a  Off-line data of glucose consumption (g/L) profile from repeated batches with defined medium RBD2-1 (□-□), RBD2-2 (●-●), and RBD2-3 (△-△).

Figure 3.6-b  Ethanol production profile (g/L) from repeated batches with defined medium RBD2-1 (□-□), RBD2-2 (●-●), and RBD2-3 (△-△).
Figure 3.6-c  Dry cell weight concentration (g/L) profiles from repeated batches with defined medium RBD2-1 (- □ -), RBD2-2 (- • -), and RBD2-3 (- △ -).

Figure 3.6-d  Optical density measurements of biomass concentration. Profiles in repeated batches with defined medium RBD2-1 (- □ -), RBD2-2 (- • -), and RBD2-3 (- △ -).
Figure 3.6-e  Total protein concentration (U/mL) Profiles in repeated batches with defined medium RBD2-1 (- □ -), RBD2-2 (- • -), and RBD2-3 (- △ -).

Figure 3.6-f  Total protein concentration (mg/mg DCW) Profiles in repeated batches with defined medium RBD2-1 (- □ -), RBD2-2 (- • -), and RBD2-3 (- △ -).
Figure 3.6-g  ADH enzyme activity (U/mL) profiles from repeated batches with defined medium RBD2-1 (- □ -), RBD2-2 (- ● -), and RBD2-3 (- Δ -).

Figure 3.6-h  ADH enzyme activity (U/gDCW) profiles from repeated batches with defined medium RBD2-1 (- □ -), RBD2-2 (- ● -), and RBD2-3 (- Δ -).
Figure 3.6-i  ADH enzyme activity (U/mg protein) profiles from repeated batches with defined medium RBD2-1 (- □ -), RBD2-2 (- ● -), and RBD2-3 (- △ -).

Figure 3.6-j  G6PDH enzyme activity (U/mL) profiles from repeated batches with defined medium RBD2-1 (- □ -), RBD2-2 (- ● -), and RBD2-3 (- △ -).
Figure 3.6-k  G6PDH enzyme activity (U/mL) profiles from repeated batches with defined medium RBD2-1 (- □ -), RBD2-2 (- ▲ -), and RBD2-3 (- △ -).

Figure 3.6-l  G6PDH enzyme activity (U/mg protein) profiles from repeated batches with defined medium RBD2-1 (- □ -), RBD2-2 (- ▲ -), and RBD2-3 (- △ -).
Figure 3.6-m  MDH enzyme activity (U/mL) profiles from repeated batches with defined medium RBD2-1 (□ -), RBD2-2 (• -), and RBD2-3 (△ -).

Figure 3.6-n  MDH enzyme activity (U/g DCW) profiles from repeated batches with defined medium RBD2-1 (□ -), RBD2-2 (• -), and RBD2-3 (△ -).
Figure 3.6-o  MDH enzyme activity (U/mg protein) profiles from repeated batches with defined medium RBD2-1 (-□-), RBD2-2 (-●-), and RBD2-3 (-△-).

Figure 3.6-p  Hexokinase enzyme activity (U/mL) profiles from repeated batches with defined medium RBD2-1 (-□-), RBD2-2 (-●-), and RBD2-3 (-△-).
Figure 3.6-q Hexokinase enzyme activity (U/g DCW) profiles from repeated batches with defined medium RBD2-1 (-□-), RBD2-2 (-●-), and RBD2-3 (-△-).

Figure 3.6-r Hexokinase enzyme activity (U/mg protein) profiles from repeated batches with defined medium RBD2-1 (-□-), RBD2-2 (-●-), and RBD2-3 (-△-).
Figure 3.6-s Pyruvate (g/L) profiles from repeated batches with defined medium RBD2-1 (-□-), RBD2-2 (-●-), and RBD2-3 (-△-).
3.2.4 Analysis of repeated batch fermentation RBD3

RBD3 is a repeated batch culture with defined medium consisting of four single batches RBD3-1, RBD3-2, RBD3-3, and RBD3-4. First batch (RBD3-1) of this experiment was discarded since the data acquisition system had failed before taking any off-line sample from RBD3-1. The CO₂ exit gas from mass spectrometer was 6.1, which usually corresponded to the first CO₂ peak, and off-line analysis of broth samples from this batch would have either started at the peak or very close to the peak. The Real Time Data Acquisition System was reinitialised and the fermenter was refilled to start a RBD3-2. Inoculum to other batches was 500 mL of the broth from the previous batch (Figure 3.7-a,b).

A difference in the amplitude of the CO₂ exit gas profile was observed in RBD3 to profiles obtained from single batch cultures, preliminary repeated batch cultures, and RBD2. Online data from RBD3, however, showed the same DOT profile for RBD3 as previous experiments. The error was considered to have been caused by the mass spectrometer instrument. Further investigations proved that the gas cylinder containing calibration gas to the mass spectrometer had been emptied. A new container was connected to the mass spectrometer and the instrument was recalibrated in phase two of RBD3-3 (Figure 3.7-a).

Figure 3.7-a shows the normalised CO₂ exit gas analysis data with arbitrary units from RBD3. Function 'MAX(A1:A2)' within Microsoft Excel spread sheet was used to identify the maximum CO₂ data point which corresponded to the first CO₂ peak in the profile. CO₂ exit gas analysis was then divided by the maximum value identified from 'MAX(A1:A2)'. These data are referred to as the normalised CO₂ data.

Comparison of the DOT profiles in Figure 3.7-b with CO₂ exit gas analysis profile (Figure 3.7-a) indicates that the mass spectrometer being out of calibration had affected the amplitude of the CO₂ data not the width of the profile.

Figure 3.7-b shows a longer time between the first refill and the trough in RBD3-2 than that of RBD3-3 and RBD3-4. Likely explanations of this observation are the inaccuracy of the inoculum size from manual refilling the fermenter and also refilling the fermenter at different phases of CO₂ exit gas profile. The inoculum variations for RBD3 are considered to be less than those observed in RBD2 (250 mL) due to a larger inoculum for the RBD3 fermentation (500 mL).
Figure 3.7-a A graphical presentation of normalised CO₂ exit gas profile in repeated batch culture RBD3.

Figure 3.7-b A graphical presentation of DOT profile in repeated batch culture RBD3.
Figures 3.7-a and 3.7-b show that the inoculum was taken at a different phase for different batches of RBD3. Inoculum for RBD3-2 contained cells from late phase 1 of RBD3-1. RBD3-3 was started with biomass from phase 5 of RBD3-2 (Figure 3.6-a) where biomass had fully utilised the ethanol inside the fermenter. For the last batch RBD3-4, the inoculum was taken about four hours after phase 7 of RBD3-3. Therefore inoculum to RBD3-2 contained much younger cells than the inoculum to RBD3-3 and RBD3-4 (after phase 5 and phase 7). In phase 1 the biomass was in the first exponential growth phase on glucose, whereas after phase 7 the biomass had totally used the ethanol and had started lysing. With reference to this explanation the time taken from refill to phase two is expected to be shorter in experiment RBD3-2, due to the younger inoculum in RBD3-2. A reasonable to assumption would be that the change in the time length of the profile would be more likely to be caused by inoculum size variations introduced by manual refilling of the fermenter.

Original data for RBD3 are presented in appendix 5. phases 1 to 4 are clearly identified for each batch. For ease of visual comparison, individual components of each batch are plotted on the same graph (Figures 3.8, a-s). Relative fermentation times were calculated on the first CO₂ peak of the profile of each batch as described in section 3.1.4. Logarithmic plots are used to linearise the data before and after the CO₂ peak of the profile. A similar vertical axis scale is chosen to those of single batch cultures and RBD2 for ease of visual comparison.

**Glucose consumption**

Glucose consumption profiles are illustrated in Figure 3.8-a. Glucose was completely used in phase 2 of the CO2 profile. Figure 3.8-a indicates the same degree of reproducibility of the profile levels as RBD2 fermentation. Visual comparison of glucose profiles from repeated batch cultures the RBD2 and RBD3 show better reproducibility for the profile of this compound than with the single batch fermentations.

**Ethanol production**

Ethanol production profiles are shown in Figure 3.8-b. An accumulation of up to 9 g/L ethanol was observed for single batches of RBD3. RBD3-3 and RBD3-4 show a maximum concentration of ethanol at relative time zero. RBD3-2, however, shows a maximum concentration of ethanol at relative time 2 hours. Ethanol profiles in RBD3 show less variation for the sample analysis than RBD2 (Figure 3.6-b) and single batch cultures (Figure 3.4-b). The most likely explanation for this observation is the variability
of the off-line sample analysis observed with ethanol assays. Off-line samples prepared for HPLC analysis of RBD3 fermentation were kept frozen for a much shorter time relative to previous fermentations.

**Biomass production**

Dry cell weight and optical density measurements are presented in Figures 3.8-c and 3.8-d. Both figures show a lower concentration of biomass (4 g/L) for RBD3-2 than the 5 g/l biomass concentration in RBD3-3 and RBD3-4. Similar biomass profiles from RBD2 (Figures 3.6-c,d) showed much better reproducibility of the biomass concentration within a repeated batch culture.

**Total protein concentration**

Total protein concentration profiles for RBD3 are shown in Figures 3.8-e,f. A much better reproducibility of these profiles are observed for RBD3 fermentation than RBD2 (Figures 3.6-e,f) and single batch fermentations (Figures 3.4-e,f).

**Intracellular enzyme activity**

Figures 3.8,g-r illustrate the intracellular enzyme activity profiles of RBD3. Enzyme activities are presented in U/mL, U/g biomass, and U/mg protein. Activities presented in U/mL show less variability than U/g biomass and U/mg protein profiles.

ADH activity profiles are shown in Figures 3.8,g-i. Visual comparison of ADH activity profiles from RBD3 indicates less batch to batch variability than that observed in ADH profiles of RBD2 (Figure 3.6,g-i) and single batch fermentations (Figures 3.4,g-i).

G6PDH activity levels are presented in Figures 3.8,j-l. Although RBD3-4 has shown a higher activity level in Figure 3.8-j, Figures 3.8,k-l represent a better reproducibility for the profile of this enzyme than RBD2 and single batch fermentations.

MDH activity level for RBD3 is shown in figures 3.8,m-n. A change in the activity level at relative time zero is clearly observed. Figures 3.8,m-n indicate low activity of this enzyme before relative time zero.

Hexokinase profiles are presented in Figure 3.8,p-r. Visual comparison of the profiles indicate that similar reproducibility is observed for this enzyme in RBD3,RBD2, and single batch cultures.
**Pyruvate production**

Figure 3.8-s shows the production of pyruvate in repeated batch RBD3. Maximum concentration of this compound (0.12 g/L) was reached at relative time zero. The first batch of this fermentation showed more variability of pyruvate in samples before relative time zero.

In this section, the reproducibility of intracellular enzyme activity levels has been demonstrated. Visual comparison of repeated batch culture graphs showed similar off-line sample profiles to those from single batch experiments. Process variations observed with data correlations in single batch fermentations were greatly reduced in repeated batch fermentations. This reduction has led to a better reproducibility of the off-line profile levels in repeated batch experiments (Figures 3.4 and 3.8). The most likely explanation of this observation is the closer similarities in inoculum age and medium preparation of the repeated batch cultures. Excellent reproducibility of CO$_2$ exit gas profiles of repeated batch cultures of *S.cerevisiae* aerobically grown on a defined liquid medium in a fully automated high performance bioreactor is also reported by Locher *et al.* (1991).

Although the aim of this work was to correlate intracellular enzyme products with CO$_2$ exit gas profile, comparison of the off-line sample profiles from RBD2 was based on DOT online data. Good reproducibility of the off-line profiles based on DOT profiles were obtained indicating that DOT data can also be used for process decision making. However, if a mass spectrometer is available, CO$_2$ exit gas data are more reliable for process decision making than DOT data. The Mass spectrometer is automatically calibrated outside the fermenter, eliminating any contamination risk. Even with mass spectrometer out of calibration in experiment RBD3, CO$_2$ profile could nevertheless be used for comparison of the data.

As a comparison with the defined medium fermentations, complex medium cultivations (RBC1 and RBC2) were performed and analysed. On-line and off-line profiles of complex medium fermentations were used to investigate the correlation of these data for complex medium cultures under the same fermentation conditions as the defined medium fermentations. Reproducibility of the profiles and enzyme activity levels of the off-line samples are then compared at different phases of CO$_2$ exit gas profile in section 3.3.
Figure 3.8-a Off-line data of glucose consumption (g/L) profile from repeated batches with defined medium RBD3-2 - □ -, RBD3-3 - • -, and RBD3-4 - △ -.

Figure 3.8-b Ethanol production profile (g/L) from repeated batches with defined medium RBD3-2 - □ -, RBD3-3 - • -, and RBD3-4 - △ -. 
Figure 3.8-c  Dry cell weight concentration (g/L) profiles from repeated batches with defined medium RBD3-2 - □ -, RBD3-3 - ● - , and RBD3-4 - △ - .

Figure 3.8-d  Optical density measurements of biomass concentration. Profiles in repeated batches with defined medium RBD3-2 - □ -, RBD3-3 - ● - , and RBD3-4 - △ - .
Figure 3.8-e  Total protein concentration (U/mL) profiles in repeated batches with defined medium RBD3-2 - □ -, RBD3-3 - ● -, and RBD3-4 - △ -.

Figure 3.8-f  Total protein concentration (U/mg DCW) profiles in repeated batches with defined medium RBD3-2 - □ -, RBD3-3 - ● -, and RBD3-4 - △ -.
Figure 3.8-g  ADH enzyme activity (U/mL) profiles from repeated batches with defined medium RBD3-2 - □ - , RBD3-3 - ● - , and RBD3-4 - △ - .

Figure 3.8-h  ADH enzyme activity (U/gDCW) profiles from repeated batches with defined medium RBD3-2 - □ - , RBD3-3 - ● - , and RBD3-4 - △ - .
Figure 3.8-i  ADH enzyme activity (U/mg protein) profiles from repeated batches with defined medium RBD3-2 - □ -, RBD3-3 - ● -, and RBD3-4 - △ -.

Figure 3.8-j  G6PDH enzyme activity (U/mL) profiles from repeated batches with defined medium RBD3-2 - □ -, RBD3-3 - ● -, and RBD3-4 - △ -.
**RBD3 'comparison graphs'**

**Figure 3.8-k**  G6PDH enzyme activity (U/g DCW) profiles from repeated batches with defined medium RBD3-2 - □-, RBD3-3 - •-, and RBD3-4 - △-.

**Figure 3.8-j**  G6PDH enzyme activity (U/mg protein) profiles from repeated batches with defined medium RBD3-2 - □-, RBD3-3 - •-, and RBD3-4 - △-.
Figure 3.8-m  MDH enzyme activity (U/mL) profiles from repeated batches with defined medium RBD3-2 - □ -, RBD3-3 - ● -, and RBD3-4 - Δ -.  

Figure 3.8-n  MDH enzyme activity (U/g DCW) profiles from repeated batches with defined medium RBD3-2 - □ -, RBD3-3 - ● -, and RBD3-4 - Δ -.
Figure 3.8-0  MDH enzyme activity (U/mg protein) profiles from repeated batches with defined medium RBD3-2 - □ - , RBD3-3 - ● - , and RBD3-4 - △ -.

Figure 3.8-p  Hexokinase enzyme activity (U/mL) profiles from repeated batches with defined medium RBD3-2 - □ - , RBD3-3 - ● - , and RBD3-4 - △ -. 
Figure 3.8-q  Hexokinase enzyme activity (U/mg DCW) profiles from repeated batches with defined medium RBD3-2 - □ -, RBD3-3 - ● -, and RBD3-4 - △ -. 

Figure 3.8-r  Hexokinase enzyme activity (U/mg protein) profiles from repeated batches with defined medium RBD3-2 - □ -, RBD3-3 - ● -, and RBD3-4 - △ -.
Figure 3.8-s  Pyruvate concentration (g/L) profiles from repeated batches with defined medium RBD3-2 - □ -, RBD3-3 - • -, and RBD3-4 - △ -. 
3.3 Complex medium fermentations "A comparison"

Repeated aerobic batch cultivation of *Saccharomyces cerevisiae* in a complex medium (chapter 2, section 2.1.2.3) (RBC1 and RBC2) was carried out to investigate the correlation of on-line and off-line data under the same fermentation conditions as the defined medium fermentations (section 2.1.4).

The on-line CO2 exit gas analysis data for the first batch in RBC1-1 was not recorded from inoculation. The fermenter was refilled after reinitialising the mass spectrometer and the data acquisition system. Off-line sample analysis of RBC1-2 is described in section 3.1.1.

Repeated batch culture (RBC2) was carried out to investigate the reproducibility of the on-line CO2 exit gas profile of the complex medium fermentations (section 3.3.2). Off-line samples from the second batch of this culture (RBC2-2) were analysed to assess the reproducibility of intracellular enzyme levels obtained from RBC1-2. Results for RBC2-2 batch are shown in appendix 6.1. Analysis of the two complex medium fermentations (RBC1-2 and RBC2-2) were the same unless stated otherwise.

The initial working volume for RBC1-2 and RBC2-2 were 5.5 L and the inoculum for both batches were 1 litre of the broth from the previous batch. For RBC1-2 samples were analysed from in the middle of the first exponential biomass growth phase to the beginning of the second biomass exponential growth phase on ethanol (Figure 3.9-c). RBC2-2, however, was analysed from the mid point of the first exponential growth phase through the transition into the stationary phase of the biomass growth (Figure A 6.1-c).

Section 3.3.1 includes on-line and off-line data analysis of RBC1-2 on complex medium to investigate the existing correlation of these data as a comparison with the defined medium fermentation (SBD4, section 3.1.2). In section 3.3.2, the reproducibility of the on-line CO2 exit gas and DOT profiles in RBC2 are discussed.
3.3.1 On-line and off-line data correlations in complex medium fermentations "A comparison"

RBC1-2 and SBD4 (section 3.1.2) were chosen to compare the DOT and CO₂ exit gas profiles with off-line sample correlations in complex and defined medium fermentations.

CO₂ exit gas and DOT profiles of complex medium fermentations (RBC1-2, Figure 3.9-a) are significantly different in general configuration from those of defined medium fermentations (SBD4, Figure 3.2-a). Maximum CO₂ value in RBC1-2 is found from the on-line CO₂ exit gas analysis as described in section 3.1.4. Correlation of the on-line and off-line profiles and their comparison with the defined medium fermentation were then based on the first peak of the CO₂ exit gas analysis. For SBD4 maximum CO₂ occurred at 22.6 hours in the second phase (Figure 3.2-a) and for RBC1-2 maximum CO₂ is at 22.9 hours (Figure 3.9-a).

Figures 3.9a-j present the on-line, and off-line profiles of the monitored and measured fermentation variables in RBC1. The dashed lines between the points on the graphs help to indicate which measurement set the points belong to. There is no implication that the underlying trend represented by the data is piecewise linear. The solid line at 22.9 hours shows the first peak of the CO₂ exit gas profile. Experimental results for RBC2-2 are presented in appendix 6.1. Analysis of the two complex medium fermentations are the same unless stated otherwise.

*Glucose consumption with ethanol and pyruvate production*

Figure 3.9-b illustrates the glucose consumption with ethanol and pyruvate production in RBC1-2. Before the maximum CO₂ exit gas concentration at 23 hours, the data corresponds to the first biomass growth phase on glucose. During this phase the glucose concentration had decreased to 0.68 g/L at the CO₂ peak without the primary metabolites ethanol and pyruvate are being formed. As glucose was exhausted at 25 hours, ethanol reached a maximum concentration of 5.4 g/L at 22 hours and pyruvate accumulated to 0.52 g/L. Biomass started consuming pyruvate and ethanol as soon as the glucose was fully utilised. Pyruvate level reaches zero at 30 hours. Ethanol concentration, however, oscillated during the slow growth phase (second lag phase) of the biomass. At the
beginning of the second growth phase, a decrease in the ethanol level was observed which is shown by the last point on the ethanol profile (Figure 3.9-b) and the last point on the biomass profile (Figure 3.9-c). For SBD4 at 40 hours ethanol was fully used and biomass had reached the end of the second exponential growth phase (Figures 3.2,b-c), whereas in RBC1-2 at 40 hours ethanol was at 3.4 g/L concentration and the second growth phase of the biomass was initiated (Figure 3.2-c). Higher accumulation of pyruvate was observed for RBC1-2 (0.52 g/L, Figure 3.9-b) than SBD4 fermentation (0.1 g/L, Figure 3.2-b). Both cultures consumed the accumulated pyruvate immediately after total utilisation of glucose. Maximum ethanol production in SBD4 was 8.6 g/L, whereas RBC1-2 showed an accumulation of 5.4 g/L ethanol. In comparison with other complex and defined medium fermentations ethanol showed a batch to batch variability of 1.5 g/L in defined medium and RBC2-2 had a maximum ethanol concentration of 11.6 g/L (Appendix 6.1 Figure A6.1-b).

Broth samples from RBC1-2 were analysed using assay kits and HPLC analysis. Glucose and ethanol of the fresh samples were assayed using enzyme based kits (Glucinet, Technicon and Boehringer Mannheim GmbH). For HPLC analysis (Figure 3.9-b) samples were frozen and analysed later. The results gave a higher concentration of both ethanol and glucose in the fresh samples, using enzyme based assay kits (Section 6.2).

**Biomass and Total protein concentration**

Production of biomass (g/L) is shown in Figure 3.9-c,d. Biomass concentration has reached 4.9 g/L at the CO\(_2\) peak (23 hours, RBC1-2). In comparison with SBD4 (4.1 g/L at the CO\(_2\) peak, Figure 3.2-c), a higher concentration of biomass was obtained with the complex medium. Total protein concentration (mg/mL) followed the same profiles as the biomass (Figures 3.2-c and 3.9-c) for both cultures. Concentration of biomass stabilised after 23 hours (CO\(_2\) peak, Figure 3.9-c), yet the specific protein (g protein /g dry cell weight) was increasing (Figure 3.9-c). A significant difference was observed in the specific protein level across the CO\(_2\) peak (Figure 3.9-d). Specific protein level in RBC1-2 (0.1 0.2 g/L) was lower than that of SBD4 (0.3-0.4 g/g biomass), the profile of this variable was also different for both fermentations. In RBC1-2 specific protein level had increased after the CO\(_2\) peak, although in SBD4 the level has almost stabilised throughout the phases 2-7 after the CO\(_2\) peak.
**Intracellular enzyme activities**

Similar activity profiles for intracellular enzymes were observed in RBC1-2 (Figure 3.9,e-j) and RBC2-2 (Appendix 6.1).

Figures 3.9,e-j illustrate the profiles of intracellular enzyme activities in RBC1-2. The first point on the enzyme activity graphs indicate a higher level of the enzyme than the second point. This change is due to the first broth sample being taken from the culture at 30 minutes after the refill process. Biomass was still in the same physiological state as the cells had been in from the end of the previous batch. Therefore the time taken for the cells to adapt the new environmental conditions for changes in their physiological state is more than 30 minutes.

Figure 3.9-e indicates a rise in ADH (U/mL) activity occurred followed by stabilisation of this enzyme after the CO$_2$ peak until 27 hours when the level increased again. The ADH level reached 1.5 U/mL activity in RBD1-2 at about 38 hours, whereas for SBD4 (Figure 3.2-e) the activity level was 14 U/mL at 38 hours after inoculation. Figure 3.9-g indicates the same level of activity (U/mg biomass) for the enzyme before and after the peak until 27 hours when the ADH level rose. In terms of biomass and protein concentrations, ADH activity showed different profiles before and similar profiles after the CO$_2$ peak. In accordance with total protein concentration and biomass concentration, the defined medium fermentation (Figures 3.2-g,i) shows a higher activity of the enzyme than that of the complex medium fermentations (Figure 3.9-g,i) throughout the phases of growth. The activity level of the enzyme in SBD4 reached 3500U/gDCW and 11 U/mg protein at 38 hours. RBC1-2, however, showed an activity of 250 U/gDCW and 1.5 U/mg protein at the same time after inoculation.

MDH activity profile in RBC1-2 was similar to that of SBD4. Activity level before the CO$_2$ peak in RBC1-2 (Figure 3.9-g) is 0.5 U/mL. At 22.9 hours when the glucose was depleted, the TCA cycle was activated and the TCA cycle enzymes were derepressed, MDH activity increased to 3.5 U/mL and started accumulating in the cells (figures 3.9-e,g,i). In comparison with defined medium culture (SBD4, Figures 3.2-g,i), much higher activity levels were observed in SBD4 (1750 U/gDCW, 4 U/mg protein) than in RBD1-2 (600 U/gDCW, 3.25 U/mg protein, Figures 3.9-g,i) at 38 hours.

Hexokinase showed a similar profile in RBC2-1 (Figures 3.9-f,h,j) to that of SBD4 (Figures 3.2-f,h,j). Similar activity levels are also shown in Figure 3.9-f,h and Figure 3.2-f,h. Activity unit per mg total protein concentration was higher in RBC1-2 (1.8 U/mg
protein, Figure 3.9-j) than that in defined medium culture (Figure 3.2-j, 0.8 U/mg protein).

G6PDH showed a similar profile and similar enzyme activity levels (Figures 3.9-f,h,) as that in SBD4 (Figures 3.2-f,h). In terms of total protein concentration G6PDH exhibited a different profile in complex medium than defined medium fermentation due to the total protein concentration in complex medium fermentations. In RBC1-2 (Figure 3.9-j) the activity increased before the CO$_2$ peak and after a sudden drop enzyme activity stabilised whereas in SBD4 (Figure 3.2-j) G6PDH activity U/mg protein was increasing throughout phases 1 to 6 at 38 hours.

3.3.2 CO$_2$ and DOT profiles

Reproducibility of CO$_2$ exit gas analysis and the DOT profiles from a repeated batch culture with defined medium is discussed in section 3.2.1. Figures 3.10-a,b show the on-line profiles of RBC2 fermentation with complex medium, where the repeated batch culture consisted of four individual batches. Inoculum for the first batch was 250 mL of the culture grown in the same complex medium in a shake flask. On-line data for the first batch were recorded. After reinitialising the data acquisition system, the second batch (RBC2-2) was immediately started (chapter 2, section 2.1.6.1). This batch was sampled for off-line analysis of the broth from mid point of the first exponential growth phase of biomass through the transition into the slow growth phase. Analysis of this batch is presented in Appendix 6.1.

Figures 3.10-a,b indicate that the CO$_2$ exit gas analysis and the DOT profiles follow significantly different patterns than those obtained from defined medium fermentations (section 3.2.1, Figure 3.4). The first CO$_2$ exit gas peak was followed by a small peak in defined medium whereas in complex medium fermentations two to three small peaks can be observed after glucose depletion at the first CO$_2$ peak. The DOT pattern was consequently different for both fermentations. When there is a peak in the CO$_2$ profile a trough is observed in the DOT pattern of both defined and complex medium. An explanation of this observation is that as soon as one of the nutrients is exhausted, the biomass consumes the maximum oxygen the cells can to produce the maximum CO$_2$, shown as troughs in the DOT profile and peaks in the CO$_2$ profile.
A good reproducibility in the amplitude of the CO$_2$ peak in Figure 3.10-a is due to the larger inoculum for RBC2 than PRB1 (section 3.2.1). RBC2 had an initial working volume of 5.5 L and the volume of the broth left in the fermenter for inoculating the consecutive batch was 1 L. Better reproducibility of the profiles for RBC2-3 and RBC2-4 are also observed in Figures 3.10-a,b.

In this section on-line and off-line correlations in complex medium cultures are discussed as a comparison with the defined medium fermentations. Although intracellular enzyme products show the same profile in both defined and complex medium fermentations, lower activity levels were observed for ADH and MDH enzyme activity levels in complex medium cultures (Figures 3.9-e and 3.2-e).

Hexokinase and G6PDH showed similar activity levels for growth on complex and defined medium, whereas, MDH and ADH had higher activity levels in defined medium. At a fermentation time, of 38 hours, ADH showed 14 U/mL activity in defined medium and 1.5 U/mL activity in complex medium. MDH activity at 22.9 hours was 3.5 U/mL for growth in defined medium and 0.3 U/mL for growth in complex medium. Since the same strain of yeast was used for growth in both media, a possible explanation of the differences in the enzyme activity levels is that cells have modified a cellular mechanism to adapt to the new environment in complex medium, indicating the flexibility of the cells for survival. Zimmermann (1992) stated that enzymatic machinery for carbon metabolism changes depending on the carbon source available. A convenient carbon source for ethanol production was considered to repress the synthesis of enzymes involved in gluconeogenesis and respiration (Zimmermann, 1992). Complex medium is richer in nutrients and the strategy of the cell is to make more cells but not necessarily keep the enzyme synthesis functioning. With defined medium, however, cells are under environmental strain and the yeast follow a different strategy for survival. Therefore the yeast would have a different metabolism to the cells grown on complex medium.

Repeated batch cultures show good reproducibility of both on-line and off-line measurements in complex medium fermentations. Single batch fermentations, however, are expected to show more batch to batch variability in complex medium than in defined medium cultures, due to variability in the complex medium components. Reproducibility of DOT and CO$_2$ profile indicates that these on-line profiles can be used to make decisions on the process for timing of the intracellular product yield and automation of the process whether the cultivation is on defined or complex medium.
Figure 3.9-a CO₂ exit gas profile - - - and DOT profile - - - of repeated batch culture 'RBC1-2' on complex medium. The solid line at 22.9 hours represent the first peak of the CO₂ exit gas profile.

Figure 3.9-b Off-line data of glucose - ● - , ethanol - ▲ - and pyruvate - ◊ - profile in repeated batch RBC1-2 with complex medium. The solid line at 22.9 hours represent the first peak of the CO₂ exit gas profile.
Figure 3.9-c  Dry cell weight concentration (mg/mL) - □ - and total soluble protein content (mg/mL) - ■ - analysis of repeated batch fermentation RBC1-2 in complex medium. The solid line at 22.9 hours represent the first peak of the CO₂ exit gas profile.

Figure 3.9-d  Dry cell weight concentration (g/L) - □ - and total soluble protein content (g/gDCW) - ■ - analysis of repeated batch fermentation RBC1-2 in complex medium. The solid line at 22.9 hours represent the first peak of the CO₂ exit gas profile.
Figure 3.9-e Off-line assay of Alcohol dehydrogenase - △ - (U/mL) and Malate dehydrogenase - ▼ - (U/mL) in repeated batch fermentation RBC1-2 with complex medium. The solid line at 22.9 hours represent the first peak of the CO₂ exit gas profile.

Figure 3.9-f Glucose-6-phosphate dehydrogenase activity levels (U/mL) - ▼ - and hexokinase enzyme activity level (U/mL) - O - in repeated batch fermentation RBC1-2 with complex medium. The solid line at 22.9 hours represent the first peak of the CO₂ exit gas profile.
Figure 3.9-g Off-line assay of Alcohol dehydrogenase -△- (U/gDCW) and Malate dehydrogenase -▼- (U/gDCW) in repeated batch fermentation RBC1-2 with complex medium. The solid line at 22.9 hours represent the first peak of the CO$_2$ exit gas profile.

Figure 3.9-h Glucose-6-phosphate dehydrogenase activity levels (U/gDCW) -▼- and hexokinase enzyme activity level (U/gDCW) -○- in repeated batch fermentation RBC1-2 with complex medium. The solid line at 22.9 hours represent the first peak of the CO$_2$ exit gas profile.
RBC1-2 data correlation

Figure 3.9-i Off-line assay of Alcohol dehydrogenase - △ - (U/mg protein) and Malate dehydrogenase - ▽ - (U/mg protein) in repeated batch fermentation RBC1-2 with complex medium. The solid line at 22.9 hours represent the first peak of the CO₂ exit gas profile.

Figure 3.9-j Glucose-6-phosphate dehydrogenase activity levels (U/mg protein) - ▽ - and hexokinase enzyme activity level (U/mg protein) - ◊ - in repeated batch fermentation RBC1-2 with complex medium. The solid line at 22.9 hours represent the first peak of the CO₂ exit gas profile.
Figure 3.10-a CO₂ exit gas profile in repeated batch culture (RBC2) with complex medium.

Figure 3.10-b DOT profile in repeated batch culture (RBC2) with complex medium.
3.4 Summary

In this chapter the following is demonstrated.

i) The correlations between on-line CO$_2$ exit gas profiles in an aerobic batch culture of *S. cerevisiae* grown on defined medium and profiles of operational parameters from off-line samples are reproducible. Reproducible correlations indicate that the process decision making of harvesting intracellular enzymes can be based on the CO$_2$ exit gas profiles.

ii) The harvesting time for a particular enzyme product depends entirely on the metabolism and intracellular enzyme activity levels. ADH enzyme activity appears to reach a maximum level after the first CO$_2$ peak. Therefore, the first peak of the CO$_2$ profile can be chosen as a signal to identify the timing of the harvesting of ADH, whereas MDH is at a maximum at the end of growth on ethanol indicating that the timing of the second peak is a more reasonable indication for the harvesting time of this particular product.

iii) The repeated batch culture technique for the aerobic batch culture fermentations of *S. cerevisiae* grown on a defined medium helped to reduce the variabilities observed in the off-line profile levels of single batch fermentations.

iv) The complex liquid medium aerobic batch fermentations with the same strain of *S. cerevisiae* under the same environmental and fermentation conditions showed different detailed profiles for DOT and CO$_2$ on-line variables in comparison with the results for the chemically defined liquid medium aerobic batch cultures.

v) Although similar on-line profile and off-line profile correlations were observed for defined and complex medium fermentations, off-line profile levels in complex medium fermentations were different due to the different metabolism of yeast cells grown on complex medium.

In the next chapter measurement variabilities of off-line sample analysis presented in this chapter are discussed.
4.0 MEASUREMENT VARIABILITY ANALYSIS

Introduction

A knowledge of measurement error is often required when measurements are used in quantitative applications. Assay variability is important for fermentation process operation and control, and for validating the models used in bioprocess simulation.

Measurement variability of the off-line assays depends on the measured concentration and could be presented as a standard deviation proportional to the measured value. An assessment of the variability of off-line sample measurements is reported in this chapter. This information can be used to establish the validity of correlations and events observed in the batch and repeated batch fermentation processes (chapter 3).

Stone et al. (1992) reported the values of the variabilities in terms of standard deviation, for two methods of dry weight analysis of microbial biomass, over the dry weight concentration range of 3-35 gL\(^{-1}\). 250 mL of broth available from a shake flask culture was used to produce forty dry weight measurements. A minimum 20 mL of the broth was transferred into 10 universal bottles. Dry weight measurements were obtained by pipetting 5 mL of well mixed broth samples on to a membrane filter. The first method involved grouping the four 5 mL measurements available from a single universal bottle, the mean of the four being taken as the representative dry weight measurement, thus producing ten measurements per broth source for assessment of measurement variability. In the second method, grouping in terms of universal bottles was ignored, each analysis was treated separately, and forty measurements per broth source for assessment of measurement variability were produced. For both methods the variations were found to be remarkably low.

Many authors have studied the production of glycolytic enzymes in *S. cerevisiae*. Schaaff et al. (1989) studied overproduction of eight different enzymes in a strain of genetically engineered *S. cerevisiae* to determine the effect of such enzymes on the rate of ethanol formation. Postma et al. (1989) examined the effect of organic acids on ethanol production of *S. cerevisiae* in a chemostat culture. In physiological studies of *S. cerevisiae*
Verduyn et al. (1990) analysed some enzyme activities and product formation. Maitra and Lobo (1971) reported the response of the specific activity and synthesis of many glycolytic enzymes after the addition of a carbon source to a hybrid yeast culture. None of these authors gave a systematic survey of the repeatability of the assay although in the latter study (Maitra and Lobo, 1971) the authors stated that the assay was completed in nine hours and that all except the alcohol and acetyl hydrogenase were "fairly stable". Bradford, (1976) gave an estimation of the variability of a soluble protein assay using three replicate measurements on the samples.

The HPLC equipment used for this work is that employed by Turner (1992). The variability of a galactose sample (about 2 g.L\(^{-1}\) solution) using the autosampler (LDC Milton Roy) is reported (Turner, 1992) where the solution was pipetted into several HPLC vials and assayed at three consecutive injections per vial. Although the majority of injections were repeated to show good reproducibility, a number of rough vials (where the sample was not injected properly) were responsible for the overall variability of this sample. The associated standard deviation for the mean value of 1.7922 g.L\(^{-1}\) of the galactose solution with all injections made was 0.522 g.L\(^{-1}\). Injections, excluding those from rough vials, gave a standard deviation of 0.032 g.L\(^{-1}\) at a mean concentration of 2.050 g.L\(^{-1}\).

Replicate sets of measurements from fermentation broth samples were used to identify the sample variation for protein and intracellular enzyme activities. For biomass optical density measurements replicates are taken from appendix 1. For glucose, ethanol and pyruvate however, samples were made up within the concentration range of the component in the fermentation broth and assayed with the HPLC. Nine replicate measurements were made for each sample for the HPLC assays.
4.1 Statistical methods

Each set of replicate measurement (one set for each broth sample) was examined to identify outliers at trends. Points were eliminated when a specific cause for a deviation was noted during the experimental procedure. The mean value of each set of replicate measurements and the associated measurement variability were calculated. The measurement variability was expressed as the standard deviation, $s$, taken as the square root of the variance of the replicate measurements, $s^2$. The relationship between the mean value and the associated measurement variability was investigated for either a proportional component or a fixed value of the variability.

Let the replicate measurements at a particular concentration be $x_i$, $i=1$ to $n$. These measurements are random samples from a population with mean value $\bar{x}$ and variance $\text{var}(x) = \sigma^2$. The variance calculated from $n$ replicate measurements is:

$$s^2 = \frac{1}{n-1} \sum_{i=1}^{n} (x_i - \bar{x})^2,$$ \[\text{where } \bar{x} = \frac{1}{n} \sum_{i=1}^{n} x_i\]

The expression for the sample variance, $s^2$, provides an unbiased estimate of the population variance $\sigma^2$ while $\bar{x}$, the sample mean, is an estimate of the population mean, $\bar{x}$. The standard deviation of the replicates, $s$, is calculated as the square root of $s^2$.

The summary statistics of replicate measurements are shown in Figures 4.1 to 4.5. The horizontal axis in the figures shows the mean value and the vertical axis shows the standard deviation. For example the point marked with an arrow on Figure 4.1 shows summary statistics for 10 replicate measurements. The point is plotted so that the mean of the 10 measurements is on the horizontal axis and the standard deviation of the 10 replicate measurements is on the vertical axis. The straight lines on the figures show the best-fit proportional relationship between standard deviation and mean value.

The gradient of the best-fit line reflects the percentage error of a mean of a sample. Therefore an estimate can be made of the error for other processed samples. For example where the slope of the best-fit proportional line for ADH is 0.054, the standard deviation expressed as a percentage mean value for this measurement is $\pm$ 5.4%. For an enzyme activity measurement of 15 U/mL, the estimated error is $\pm$ 0.8 U/mL. When the relationship between the standard deviation and the mean measured values is not proportional (e.g. dry cell weight measurements, Stone et al. (1992), and glucose at concentrations above 17g/L (Figure 4.5)), a fixed component of variability is considered.
4.2 Production of broth samples for protein and enzyme assays

Off-line laboratory measurements required manual sampling of the fermentation broth, a cell disruption stage, a manual dilution and division of each sample into multiple subsamples for the replicate measurements. Between 25 and 35 replicate measurements were made on each broth sample for the ADH and soluble protein assays. Other enzyme assays used 10 replicates, except hexokinase where the response time of the assay restricted the practical number of replicates to three.

The statistical analysis of the samples required numbers of replicate samples. Due to the length of time taken for each enzyme assay, repeated measurements for all the enzyme assays could not be obtained between sampling intervals of batch and repeated batch fermentations carried out for this work (chapter 3). To produce the materials for the statistical analysis of the soluble proteins and alcohol dehydrogenase assays, samples were taken from batch and continuous cultures (Gregory et al., 1995). The measurement variability analysis for other enzymes was carried out only on broth samples from continuous culture.

In continuous culture, a glucose concentration of 40 g.L\(^{-1}\) permitted biomass growth of up to 20 g.L\(^{-1}\). The strain of \textit{S. cerevisiae} and medium used for continuous culture were the same as those used in batch experiments. The working volume for this fermentation was 1.5 L, with medium fed to achieve dilution rates in the range of 0.06 h\(^{-1}\) to 0.28 h\(^{-1}\). The agitation and aeration rates maintained the dissolved oxygen tension about 10%. Fermentation sampling for both cultures involved samples of at least 40 mL divided into two 23mL universal bottles.

Samples were disrupted within two minutes of being taken from the fermenter vessel and kept on ice throughout the procedure. The replicate assays on the sub-samples were completed within twenty minutes of disruption, except in the case of ADH where a longer set of repeats using an autosampler took up forty five minutes. All assays were performed manually as described in chapter 2 (section 2.3) except ADH which used an in house stopped-flow system consisting of a Gilson (Luton, Beds) model 222 autosampler, two 405 syringe pumps and a DU65 spectrophotometer which allowed sequential processing of replicate measurements. The reagent and the volumes for the stopped flow system were the same as for the manual assay (Chapter 2, Section 2.3.4).
4.3 Off-line sample variability analysis

4.3.1 Variability in biomass measurement

Optical density measurement
For biomass optical density analysis, replicate measurements from appendix 1 are used to show the variability analysis of this measurement (Figure 4.1). Every point on the graph represents the summary statistics for 10 replicate measurements. The least squares fit shows the standard deviation is proportional to the replicate measurements on a single sample. From Figure 4.1, the gradient of the least squares fit is 0.006. Therefore a standard deviation of ±0.6% reflects the variations in optical density measurements.

![Figure 4.1] Statistics of the biomass optical density measurements (660 nm).

Dry cell weight measurement
Stone et al., (1992) reported the values of the variabilities in terms of standard deviation, for two methods of dry weight analysis, over the dry weight concentration range of 3-35 gL⁻¹. The sample standard deviation (gL⁻¹) against the mean dry weight concentration (gL⁻¹) showed a proportional relationship for the range of 0-10 gL⁻¹ dry weight. From the best fit proportional relationship between the standard deviation and the mean value of the grouped analysis, σ ±3.5% variability is observed in the work of these researchers.
Dry weight analysis of broth samples from batch and repeated batch cultures in chapter 3 were obtained using the same technique as the grouped analysis. Samples were also within 0-10 gL\(^{-1}\) dry weight. A standard deviation of ±3.5% is therefore assumed to be reasonable for dry weight analysis in this work.

4.3.2 **Variability in soluble protein and ADH activity measurements**

In the case of ADH and total soluble protein assays, the measurement variability analysis was carried out using samples from batch and continuous fermentations. For the soluble proteins, batch cultures gave samples in the range 0 to 4 g.L\(^{-1}\) while samples with values up to 16 g.L\(^{-1}\) were obtained from the chemostat culture. For the alcohol dehydrogenase assays, the corresponding values were 0 to 17 U mL\(^{-1}\) for the batch and 0 to 86 U.mL\(^{-1}\) for the continuous culture. The symbols used to plot the points in Figure 4.2 and Figure 4.3-b indicate points where these conditions varied. All groups of points on the graphs appear to lie on the same trend, regardless of the fermentation method. This result is considered to be due to using the same precalibrated pipettes and spectrophotometers, and following a well regulated use of the standard operating procedure.

4.3.3 **Variability in replicate protein measurements**

Figure 4.2-a shows protein measurements from batch and continuous cultures. Each point on the graph represents the summary statistics for 25 repeated measurements. There is a proportional relationship between the standard deviation of the replicate measurements and the mean value; the log-log axis of Figure 4.2-b shows the proportional relationship holds over several orders of magnitude.

From Figure 4.2-a the slope of the best-fit line is 0.036. A standard deviation of ±3.6% would therefore reflect the variation to be expected for protein measurements. At ±3.6% the variability of the soluble protein assay is higher than the ±1.2% previously reported by Bradford, (1976) on three replicates of bovine serum albumin. When a larger number of replicates are used in the calculation of standard deviation (s), the measured standard deviation would be closer to the population standard deviation (\(\sigma\)). The vertical scatter in Figure 4.2-a is a reflection of the distribution of the measured standard deviation around the true value , \(\sigma\), which is given by the best-fit straight line.
Figure 4.2-a  Statistics of soluble protein assay. Broth samples were from batch (♦) and continuous (○) cultures of *S. cerevisiae*. Each point shows the standard deviation and the mean value of the replicate measurement on a single broth sample. The least squares fit shows that the standard deviation is proportional to the measured value.

Figure 4.2-b  The log-log plot of statistics of soluble protein assay. This plot shows that the proportional relationship holds over several order of magnitude.
4.3.4 Variability in replicate ADH measurements

The variation of ADH activity for replicate measurements of a single selected broth sample is illustrated in Figure 4.3-a. ADH activity measurement was repeated 25 times, shown on the horizontal axis. The horizontal axis can also be regarded as a time axis; the replicate measurements being spaced by about 2.9 minutes. Replicate measurements were always 2.5 to 3 minutes apart, and showed an initial rapid activation followed by a slower reduction in activity as the homogenised broth sample aged. This pattern was observed for all ADH assays regardless of whether the assays were carried out manually or automatically.

The statistics of ADH measurement by autosampler from batch SBD4 and the continuous culture are shown in Figure 4.3-b. The least squares fit line shows a proportional relationship between the standard deviation and mean values. A value of ±5.4% is observed for the proportionality relationship in ADH.

The use of standard deviation in a case where data exhibits a trend needs justification since both the trend and random variability leads to a spread of values in the replicate measurements.

The replicate ADH measurements on a single broth sample exhibited a trend characterised by an initial rapid rise in activity followed by a more gradual decline. A longer sequence of repeated measurements would therefore be expected to have a higher standard deviation than shorter runs. Measurements taken immediately after the homogenisation step would intercept the initial rise in the activity and show a greater variability than the measurements taken when the enzyme activity is following a more gradual decline.

The aim of determining the standard deviation of the replicate was to be able to evaluate a standard deviation error for the measurement. The ±5.4% error would seem to be an overestimate because this value would include the systematic effect of the decay in enzyme activity. But if the timing of the measurement is an uncertain parameter and is subjected to random variation, then a standard deviation of ±5.4% would reflect the variation to be
expected. The procedure adopted is a reasonable one for this work, since the timing of the measurements is uncertain.

An autosampler was used to measure the ADH activity levels of the replicate samples reported in this chapter. Although the reagents and the volumes for the stopped flow system were the same as for the manual assay, in using the autosampler the manual pipetting of the reagent (2.9 mL) was eliminated. The error associated with the use of the autosampler for the ADH assay is therefore considered to be less significant than the error associated with the use of manual pipetting of the reagent.

Stone et al., (1992) examined the pipetting error in the studies for dry weight measurement variability analysis, where two methods of analysis were investigated. The ungrouped method of analysis involved the sample being pipetted four times. The grouped method did not involve the manual pipetting step. A lower value of variability associated with the method of dry weight analysis used in a grouped analysis was reported compared with the ungrouped analysis which showed pipetting contributed to the variability. A ±0.5% difference was observed in the error analysis of the two methods in the linear region of 0-10 g/L dry cell weight.
Figure 4.3-a  Replicate measurements of ADH activity from a single broth sample. The results indicate an initial rise followed by a slower decline. The horizontal axis can be regarded as a time axis; the replicate measurements are spaced by about 2.9 minutes.

Figure 4.3-b  Statistics of ADH assay. Broth samples were from batch (♦) and continuous (○) cultures of *S.cerevisiae*. Least squares fit shows the proportionality of the standard deviation to the measured values.
4.3.5 Variability in MDH, hexokinase, and G6PDH replicate measurements

Figure 4.4 shows the standard deviation verses mean plots for the assays of intracellular enzymes, MDH, G6PDH, and hexokinase. In Figure 4.4 every point on the G6PDH and MDH figures represents summary statistics for 10 repeated measurements and for hexokinase assay each point is the summary statistic of three replicate measurements. The standard deviation is proportional to the mean values in all cases. The straight lines on the figures show the best-fit proportional relationship between standard deviation and mean value. From the slope of the best fit line proportional relationship, expressions for the standard deviations are derived and listed in Table 4.1.

4.3.6 Variability in glucose, ethanol, and pyruvate replicate measurements

Figure 4.5 shows the summary statistics of replicate measurements. Every point on the graph indicates 9 replicate measurements at a mean concentration. Standard deviation verses mean plots for the glucose, ethanol, and pyruvate HPLC analysis are shown (Figure 4.5). An over estimate of the standard deviation for a measurement with a mean value on the x-axis is given by the dashed line in the case of glucose and ethanol assays. Stone et al., (1992) reported a similar variability profile in their dry weight measurement studies. For glucose assays of up to 17 g/L glucose concentration a proportional relationship was observed between standard deviation and mean values. From the best fit proportional relationship, variability of the glucose assay in this region is ±0.3%. Samples with a concentrations of more than 17 g/L are assumed to have an upper bound (maximum) standard deviation of 0.053 g/L.

Best fit proportional relationship was applied to ethanol samples of up to 4.2 g/L concentration. The ±0.6% variability was observed in this range of concentrations. Samples with higher concentrations than 4.2 g/L were assumed to have a standard deviation of 0.025 g/L. For pyruvate assay, however, standard deviation is proportional to the mean value of the replicate samples. Due to a very low standard deviation for the pyruvate assays, either an overestimate of 0.0035 g/L as a fixed component of variability can be considered, or from the equation of the best-fit proportional relationship the standard deviation can be calculated from:

\[ \sigma = 0.0026 \bar{x} + 0.008 \text{ g/L} \]
Figure 4.4  Statistics of additional intracellular enzyme assays. The samples were from continuous culture of <i>S.cerevisiae</i>. Standard deviation is proportional to the mean value in each case.
Figure 4.5  Statistics of HPLC analysis. An over estimate of the standard deviation for a measurement with a mean value on the x-axis is given by the dashed line in the case of glucose and ethanol assays. For pyruvate assay, however, standard deviation is proportional to the mean value.
4.4 Summary

The measurement variability of the assays must be assessed to validate the correlation of off-line assay profiles with the on-line monitored profiles of the fermentation variables. This chapter provides an estimation of the measurement variability analysis in terms of standard deviation of the off-line measured samples.

From the graphs of standard deviation versus the mean value, a best-fit proportional relationship for the replicate measurements was obtained. From the gradient of the best-fit proportional line, standard deviations as a % of the mean value are summarised in Table 4.1. In the case of glucose, and ethanol an upper bound (maximum) standard deviation is listed in Table 4.1.

<table>
<thead>
<tr>
<th>Assay</th>
<th>standard deviation as % of mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD</td>
<td>±0.6%</td>
</tr>
<tr>
<td>Dry weight</td>
<td>±3.5%</td>
</tr>
<tr>
<td>(Stone et al., 1992)</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>±3.6%</td>
</tr>
<tr>
<td>ADH</td>
<td>±5.4%</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>±3.0%</td>
</tr>
<tr>
<td>G6PDH</td>
<td>±5.0%</td>
</tr>
<tr>
<td>MDH</td>
<td>±3.1%</td>
</tr>
<tr>
<td>Glucose</td>
<td>±0.053 g/L</td>
</tr>
<tr>
<td>Ethanol</td>
<td>±0.025 g/L</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>±0.35%</td>
</tr>
</tbody>
</table>

Table 4.1 Standard deviation of some assays for \textit{S.cerevisiae}.

From Table 4.1 standard deviations as a % of the mean values were used to estimate the error for fermentation sample measurements. For example from Table 4.1, hexokinase standard deviation as a percentage mean is ±3.0%. An estimation of the error for a hexokinase assay with a measurement activity of 1.6 U/mL is therefore calculated by multiplying 0.03 by 1.6 which gives an error of ±0.048 U/mL.
5.0 PROCESS DECISION MAKING

Introduction

This chapter includes the methods involved in using the CO₂ exit gas monitored variable to base process decision making (e.g. the timing of feed in a fed batch culture, prediction of product formation) on calculated parameters rather than predetermined schedules for a better design, optimisation and control of fermentation processes. Distinct changes in the on-line monitored variable, CO₂ exit gas concentration are used to recognise the specific identifiable phases of glucose depletion, biomass formation and the onset of the identifiable intracellular enzyme production.

Mathematical techniques used to detect the CO₂ exit gas peaks are explained in section 5.3. On-line implementation of the peak detection is also outlined in section 5.3.3.

5.1 Intracellular enzyme production schedules

Production scheduling in fermentation processes is often based on the fermentation time starting from inoculation time zero. Correlations observed between the CO₂ exit gas monitored variable and the intracellular enzyme products (Chapter 3, section 3.1) and the good reproducibility obtained from repeated batch cultures indicated that optimal production scheduling could be based on the culture activity phases identified by the CO₂ exit gas analysis.

The batch to batch variability observed in fermentation processes is revealed by slight variations in the duration of the microbial growth phases which in turn causes variations in the duration of the CO₂ exit gas profile. Choosing CO₂ phases as reference points for further process decision making therefore eliminates the interference due to variability in time length. Consider two batch fermentations under the same environmental and fermentation conditions with different inoculum age (one with an inoculum age of 48 hours greater than the other). The fermentation with an older inoculum would be expected to have a longer lag phase (Bailey and Ollis, 1986). Even though biomass and CO₂ profiles would have different durations, the first exponential growth phase would
terminate at the first CO₂ peak for both fermentations regardless of the inoculum age difference. Therefore the operator could reasonably base the process decision making on the actual signal from the CO₂ profile rather than fermentation time from inoculation.

The first CO₂ peak indicated depletion of glucose, maximum ethanol concentration in the broth, and termination of the first biomass growth phase. Therefore, the first peak of the CO₂ profile can be used as a signal for starting the glucose feed into the fermenter for fed-batch cultures or transferring inoculum into a fermenter. Where production of intracellular enzymes are of interest, different harvest points have been identified for different products (section 3.1.3). Both the first and the last peak of the profile may be used as signals for product scheduling.

However, both process and measurement variability of fermentation processes must be considered when basing the production schedule on signals from the CO₂ profile. For process decision making in this project, variability of the biological events during fermentations was studied by investigating on-line and off-line data correlations (Chapter 3). Statistical analysis of the data was also performed using repeated measurements of the samples from batch and continuous cultures to investigate the accuracy and to examine the reliability of the off-line measurements, (chapter 4).

For ADH production scheduling, in the first biomass growth phase when the cells are consuming glucose before the first CO₂ peak, ADH appeared to have a higher production rate than in the second biomass growth phase when the biomass is consuming ethanol (Figure 3.3-a). ADH had reached 17 U/mL at the first CO₂ peak and stabilised for 8 hours in phases 3 and 4 (Figure 5.1, 23.5-35 hours). As the cells terminated biomass growth on ethanol, the ADH level had reached 27 U/mL (Figure 5.1, 40 hours). Thus in the first 24 hours of the fermentation, 17 U/mL ADH was produced but a further 18 hours led to a total product concentration of 27 U/mL. The optimum point of harvesting for ADH as a product could then be identified by considering process and measurement variability. In single batch cultures, the process variability of ADH was 2-11 U/mL (Figure 3.4-g). The process variability was greatly reduced by employing the repeated batch technique (Figure 3.8-g). Standard deviation as percentage mean of the ADH replicate samples was found to be ±5.4% (Chapter 4, Table 4.1). Therefore, ADH activity measurement of 17 U/mL has an error of ±0.918 U/mL and enzyme activity of 27 U/mL carries a measurement error of ±1.458 U/mL.
The errors are sufficiently small that the trends can be identified reliably and the proposed decision making based on the CO$_2$ exit gas profile is feasible. Hence, both the first CO$_2$ peak and the last CO$_2$ peak (Figure 5.1, 40 hours) can be chosen as signals for harvesting the ADH product depending on the product concentration required and the time allowed for the process.

![Figure 5.1 CO$_2$ exit gas analysis profile (---) and the ADH enzyme activity (U/mL)](image)

- o - from SBD4.

If the requirement was to harvest other enzymes, different CO$_2$ peaks can be used, as follows:

MDH activity was initially detected after the first CO$_2$ peak and the maximum level of the enzyme was observed in phase 7 of the CO$_2$ profile (Figure 3.2-e). Optimum harvest time for MDH enzyme product is at phase 7 (Figure 3.3-c).

G6PDH enzyme accumulated inside the cell until phase 6 when degradation of this product was observed (Figure 3.2-f). Therefore harvesting this product ought to be based on the first peak of the CO$_2$ profile and time (e.g. harvest cells 14 hours after the first CO$_2$ peak).

Hexokinase enzyme activity profile followed the biomass formation profile throughout the fermentation time. Optimum harvest time for this product is when biomass is at maximum concentration (Phase 7, Figure 3.2-f) indicated by the last peak of the CO$_2$ profile.
5.2 Process automation

Application of computer technology to fermentation processes has permitted better design optimisation and control of these processes. Traditionally, the operator has been responsible for monitoring the current status of the fermenter, initiating proper control commands (e.g. emptying the tank for harvesting the product, starting the feed in a fed batch culture), and also for specifying desired control parameters (set points, flowrates) when planning to start any operation - operator controlled operation. Software and computer packages available are now used to perform the task usually carried out by human function to automate process decision making.

Hai (1995) stated that operations under operator control mean that the decision making carried out is essentially an open-loop which has a number of disadvantages:

a) depending on the operator involved and the complexity of the operation, the total operation time is often longer in open-loop control than that under closed-loop control.

b) there is a considerable room for errors to be made. The number of actions undertaken by the operator is often large, and the actions may need to be taken in a certain order.

c) the safety of the operation relies on the skill and responsibility of individual operators.

d) reproducibility of the operations is low being dependent on the individual operator.

e) operator controlled operations are particularly difficult when the process involves unstable materials (e.g. G6PDH degrades in phase 5 of the CO$_2$ exit gas profile, section 3.1.2) or when a material is has to be processed immediately. In such cases, the operator has to monitor and predict the availability of the process units which may be potentially used to process the unstable material. This prediction becomes extremely difficult, even impossible, when complex operations are involved, or a large number of batches are to be processed at the same time.

This section describes the methods investigated to recognise the CO$_2$ peaks and to use the signals for further decision making of a fermentation process. Process automation such as seed transfer into fermenters, starting the feed into the tank for fed batch operations, harvesting the product whether intracellular or extracellular, and refilling the tank for repeated batch cultures are considered.
5.3 CO₂ peak detection

The correlation of the on-line exit CO₂ gas and DOT profiles with off-line fermentation data was investigated for a number of batch cultures (Chapter 3). Both on-line profiles showed good correlation with the off-line analysis. Exit CO₂ gas profile was chosen as the variable to be used for process decision making in this chapter. The CO₂ variable has a number of advantages in comparison to the DOT profile:

a) clear identifiable phases are obtained from the profile of CO₂ exit gas analysis.

b) the mass spectrometer is calibrated every 24 hours without interrupting the fermentation process whereas the DOT probe can not be rinsed and recalibrated during a repeated batch culture.

c) it is easier to recognise signals from CO₂ peak than the DOT profile for on-line control and process decision making.

The raw on-line data was stored on an IBM PC in CSV format. Data were transferred to Excel spread sheet for further analysis. Elapsed time recorded from the mass spectrometer was converted to real fermentation time based on the inoculation time zero. Occasional rogue data such as -999 or 0.00 were observed in the data which were removed from the data file before any analysis. For SBD1 and SBD2 every second data point was recorded as zero. The zero points were removed from the file by using set criteria and set database commands from the data menu in Excel.

Several techniques were used to investigate the identification of the first CO₂ exit gas peak from the profile. Linearisation of the profile was implemented to simplify the CO₂ exit gas profile for detection of the peak (section 5.3.1). A Matlab program was used to calculate the derivative of the profile although differentiating the profile would exaggerate the noise in the data (section 5.3.2). LabVIEW programming was also used to demonstrate the on-line peak recognition of the CO₂ exit gas variable during the time course of the fermentation (section 5.3.3).
5.3.1 Linearisation of CO$_2$ exit gas profile

Marshall (1992) identified linear data pieces as the most appropriate structure of fermentation data files and developed a computer program to simplify fermentation data into piecewise linear data segments. The program was used to linearise data from a single batch culture with defined medium (Figure 5.2, SBD2). Simplified profiles can be used for peak detection of the CO$_2$ exit gas analysis as shown in Figure 5.2. The program was run on a Sun Sparc Station and the result was obtained in less than five seconds.

![Figure 5.2 Piecewise linearisation of CO$_2$ exit gas data from fermentation SBD2, using the software developed by Marshall (1992). - ★ - indicates the end point of each linear data segment. - • - is the CO$_2$ exit gas profile.](image)

The program is written so that the start point of the line is the first data point and all the subsequent lines start from the last point of the previously fitted line. The program initially fits a line over one hundred data points (arbitrarily chosen). The algorithm finds the best fit through all the data points being converted, and is not constrained to pass through the data points. If the fit is good, an attempt is made to extend the line to cover a further one hundred data points. This extension step is repeated until the whole data set is covered or
a bad fit occurs. When the fit is poor, the line is extended over one more point and the best fit line is assessed. If the fit is still poor or the line has reached the end of the data set, the line is fitted over a reduced number of data points (fifty less data points). If the line is still not accepted the program fits the data by contracting by ten and expanding by one data point and finally by reducing one point at a time to obtain the best fit line.

The program gave good indication of the first CO$_2$ peak. The disadvantage of this software for CO$_2$ peak detection is that a complete data set is required to run the program and the on-line implementation of the peak detection algorithm becomes difficult. Other techniques were therefore considered.

5.3.2 Derivative of the CO$_2$ profile

The CO$_2$ exit gas profile from a single batch fermentation of $S.cerevisiae$ with defined medium (SBD2, Figure 5.3) was used to determine the first and second derivative of the data. The first signal identified would be the first maximum in the CO$_2$ exit gas profile and would indicate the glucose depletion of the broth. Timing of the harvest or refilling the tank could also be based on this signal. The same technique is shown to be applicable to the complex medium fermentation (RBC2-1, Figure 5.3).

Figure 5.3 indicates that for a defined medium fermentation, the first derivative exhibits two signals at 22 hours and 38 hours. The first signal indicates depletion of glucose and the second signal is when ethanol was completely used by the cells. Both signals are also present in the second derivative (Figures 5.3 and 5.4). The first derivative in complex medium (Figure 5.4) has displayed more noise than the first derivative of the defined medium fermentation (Figure 5.3). Complex medium fermentation has also shown two signals for the first and second derivatives, the second one being smaller than the second signal of SBD2 fermentation. This decrease was due to the nature of the original data in complex medium fermentation.

Differentiating the data has the effect of exaggerating the noise. Fermentation data files more closely spaced in time have been observed to show less noise. More frequent analysis can be achieved by connecting fewer fermenters to the mass spectrometer. The number of scans per minute from the mass spectrometer increases as fewer fermenters are
used. Therefore, for industrial fermentation where a large number of fermenters are connected to the mass spectrometer data are recorded at longer intervals causing noisier data.

The Matlab program was written in MATLAB version 4.1. The program was run on a Sun Spark Workstation. Figures 5.3 and 5.4 are from the results of the program transferred to a Macintosh computer for graphical presentation. For SBD2 fermentation the CO$_2$ exit gas data from the mass spectrometer had zero values for every other two scans. The time, however, was recorded correctly. Excel spread sheet was used to clean the data file. This cleaning led to two vectors (time and CO$_2$) containing coarsely spaced data. A new more finely spaced vector was therefore specified and using cubic spline interpolation a vector corresponding to the new vector was made. The first and second derivative of the new vector could then be calculated in the Matlab programming environment.

Both linearisation and differentiation techniques are not easily applicable on-line. With a view to on-line implementation, LabVIEW programming (Section 5.3.3) was used to investigate the capability of this technique in on-line recognition of the peak.
Figure 5.3 CO₂ exit gas profile from SBD2 fermentation with defined medium. First and second derivatives of the profile are calculated using Matlab programming environment.
Figure 5.4 CO₂ exit gas profile from RBC2-1 fermentation with complex medium. First and second order derivatives of the profile are calculated using Matlab programming environment.
5.3.3 On-line recognition of the CO₂ signal

To be useful for process automation and product scheduling signals from CO₂ exit gas data should be analysed on-line. Development in computer technology and availability of user friendly computer programs and softwares has helped on-line control and optimisation of bioprocesses.

Gregory et al. (1994) used a visual programming environment (LabVIEW) to demonstrate the development of a system to control specific growth rate in a fed-batch culture. The visual nature of the programming language and the symbolic representation of the functions as icons was reported to enabled quick programming. Gregory et al. (1994) indicated that such a programming environment is efficient for supervisory control and on-line optimisation of bioprocesses.

LabVIEW programming environment was used for on-line identification of the signals from CO₂ exit gas profile (Appendix 7). The program was written in LabVIEW version 2.1.1 (National Instruments Ltd., Newbury, UK) and was run on an Apple Macintosh IICx computer with 8 MByte RAM, 80 Mbyte hard disk and 256 colour 13 inch high resolution monitor. A VI subroutine program (Appendix 7.1, Get CO₂ data) was written for the acquisition and storage of exit gas data from the mass spectrometer where data are read in as ASCII text.

In the main program (Appendix 7.2, peak detect standalone) a signal conditioning criterion is applied over a window of data points of fixed length. The signal conditioning is a moving point average smoothing used in the program. Moving point average smoothing computes the average of a number of sequential points, replacing the centre point with the average value. This averaging has the benefit of making the first derivative calculation less noisy.

\[
(CO₂)_c = \left\{ \frac{\sum_{i=1}^{N} (CO₂)_i}{N} \right\}
\]

191
Where \((\text{CO}_2)_c\) is the calculated centre point of the interval, \((\text{CO}_2)_i\) equals the \(i\)th value of the \(\text{CO}_2\) data in the interval, and \(N\) is any number of sequential data. \(N\) is kept as a small number to avoid a time delay in the on-line procedure since time delay is \(\frac{N}{2}\).

In the peak detection program (Appendix 7.1), the number of sequential data is 3. Program shows the average moving point for \(\text{CO}_2\) and time data. The last two center points of the interval are used to find the derivatives.

\[
\frac{d\text{CO}_2}{dt} = \frac{(\text{CO}_2)_{c,i+1} - (\text{CO}_2)_{ci}}{\Delta T}
\]

where

\((\text{CO}_2)_{ci}\) is the \(i\)th value of the calculated center point of the interval and

\(\Delta T\) is the time interval.

The first signal is recognised when

\[
\frac{d\text{CO}_2}{dt} < 0
\]

since this condition implies that the \(\text{CO}_2\) peak has passed and \(\text{CO}_2\) has started to reduce.

The program was tested with cleaned data form the mass spectrometer and showed successful recognition of the signal (Figure 5.5). Although the program was not run in real time (i.e. at the time of fermentation), the program was run sequentially through the data file just as though the points were arriving in a real time sequence. Therefore from results in Figure 5.4, the procedure is acceptable for on-line detection of the \(\text{CO}_2\) peak.

Other VI sub routines were also included for on-line cleaning of rogue data form the mass spectrometer before the on-line implementation of the program.
Figure 5.5 CO\textsubscript{2} exit gas profile from SBD2 fermentation with defined medium. First order derivative of the profile is obtained from moving point average smoothing (Appendix 7.1) using LabVIEW programming environment.

5.4 Summary

In chapter 3, information from off-line data is used to discuss the intracellular enzyme production schedules. From the on-line and off-line data correlation (Chapter 3), CO\textsubscript{2} exit gas analysis is chosen in this chapter as the on-line fermentation data. Mathematical programming techniques were used to base production scheduling on calculated parameters rather than on predetermined schedules. Recognition of signals from the CO\textsubscript{2} exit gas profiles has been demonstrated. Two signals were identified from the CO\textsubscript{2} profile, and the LabVIEW programming environment was enhanced to incorporate on-line process decision making for harvesting the intracellular enzyme products.
6 CONCLUSIONS AND RECOMMENDATIONS

The objective of this thesis is to investigate the concept of using easily monitored on-line variables of aerobic batch culture fermentations, such as the CO\(_2\) content of the exit gas from the bioreactor vessel, correlated with the biological events inside the fermenter for example substrate depletion and intracellular product formation, as the control parameter of the fermentation process and as the basis for process decision making, namely the timing of feed addition in a fed batch culture and the automatic control of the culture liquid harvesting and refill operation in a repeated batch fermentation. In this work a strain of Baker's yeast, *Saccharomyces cerevisiae* (strain GB4918) from Distillers was used as the test micro-organism. The yeast was aerobically cultured in a conventional batch stirred tank fermenter, as described in chapter 2, in a chemically defined liquid medium with glucose as the carbon limiting substrate at pH 4.5 and 28 °C. An undefined liquid medium was also used for comparative studies.

The aerobic culture of yeast in relation to culture metabolism and intracellular enzyme formation is reviewed on the literature survey presented in chapter 1. The literature suggests that the CO\(_2\) content of the exit gas from the aerobic culture of yeast identifies 7 distinct phases of the cellular activity during the time course of the aerobic batch culture. Accordingly the CO\(_2\) content of the exit gas from the aerobic batch culture of the yeast was selected in this project as the on-line monitoring and control parameter- variable. Measurement methodology of the on-line and off-line culture variables are described in chapter 2 and measurement variability analysis is discussed and tested in chapter 4. The experimental results of the aerobic single batch cultures are reported and discussed in chapter 3, whilst the subsequent process decision making, based on the CO\(_2\) content of the exit gas analysis, is evaluated in chapter 5.

Variability of the biological events during an aerobic yeast fermentation process was studied by investigating on-line and off-line data correlations. The accuracy and reliability of the off-line measurements were examined using statistical analysis of the data from repeated measurements of broth samples from batch and continuous cultures. Methods involve using CO\(_2\) exit gas monitored variable to base process decision making on calculated parameters, rather than predetermined schedules, for a better design, optimisation and control of fermentation processes are also presented, such that the thesis demonstrates that the objective of this thesis was achieved.
6.1 Conclusions

On the basis of the work presented the following conclusions are made:

6.1.1 The literature survey, presented in chapter 1, identifies the two main hypothesis on the aerobic culture of yeast, namely limited respiratory capacity at the TCA cycle, see Figures 1.3, 1.4, and 1.5, and repression-derepression of the enzymes in the TCA cycle, the Pasteur-Crabtree effects. The enzymes of the interest to this project are identified in Figure 1.3 in bold italics as glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), and alcohol dehydrogenase (ADH). Intracellular enzymes, total protein and cell biomass along with extracellular glucose, ethanol, and pyruvate concentrations were monitored in the cell and cell culture environment to evaluate the function of the fermentation and respiration pathways from pyruvate in the yeast cell during aerobic batch culture. The literature indicates that the dispute between the two hypothesis continues to this date. The observation made with reference to this thesis is that repression-derepression of the fermentation-respiration pathway enzymes is a special case of limited respiratory capacity and that deactivation of for example (MDH) which lead to shut down of the TCA cycle and zero respiration is then the limiting phenomenon of the limited respiratory capacity in yeast. The proposal is that the limited respiratory capacity in yeast is variable. An obvious limiting case is the absence of oxygen and hence shut down of the TCA cycle. Whereas the TCA cycle operation is variable in the presence of oxygen and whether such variance in yeast cell energy conservation is reflected in carbon dioxide production, which in turn is the monitored on-line control parameter, is therefore the underlying aim of this project.

6.1.2 \( \text{CO}_2 \) exit gas analysis profiles from aerobic batch cultivation of *S. cerevisiae* grown on defined medium can be divided into 7 distinguishable phases on the bases of metabolic production and consumption (Locher *et al.*, 1993), as shown in Figure 1.8.

6.1.3 On-line measurement of relevant biological variables is demanding and difficult. Although on-line measurement of some fermentation variables has recently shown some success, there is still the need for reliable on-line sensors that can continuously and rapidly measure concentration of biological variables inside the fermentation broth. Reliable on-line measurement is required to base process decision making and scheduling of fermentations on events such as achievement of a certain concentration of a product.
6.1.4 The measurement variability of the assays used to validate the correlation of concentration profiles of the off-line fermentation variables with the on-line monitored variables, CO2 content in the exit gas, is important to demonstrate both the variability of the correlations and the sensitivity of the on-line variable to cellular functioning and fermentation process control. A detailed statistical analysis of the methodology of the measurement analysis is presented in chapter 4. The standard deviations as % of mean values are summarised in Table 4.1. The data in Table 4.1 provided a basis for evaluating the performance of an aerobic yeast batch culture with reference to these culture variables and clearly demonstrated the experimental limit for data sensitivity at ±6% maximum. Any variable over this maximum must therefore be significant and be due to either an operational engineering change or a change in yeast cell functioning.

6.1.5 A great amount of information is available from the on-line fermentation data. For a fermentation process to be monitored as accurately and reliably as possible, instruments should be regularly examined and calibrated to obtain reproducible measurements. When homogenisation of broth samples is involved in off-line analysis, samples must be homogenised and analysed as quickly as possible. For the intracellular enzymes discussed in this work, broth samples were frozen (using liquid nitrogen) both as whole cells and also after homogenisation. Samples did not show any activity after being kept at -70°C for two weeks. Development of an on-line homogeniser would greatly help the process of monitoring and control of fermentations.

6.1.6 Using the repeated batch culture technique for fermentations of *S.cerevisiae* grown on defined medium helped in reducing the variabilities observed in the off-line profile levels of single batch fermentations as discussed in chapter 3. Whilst Complex medium fermentations with the same strain of *S.cerevisiae* under the same environmental and fermentation conditions showed different detailed profiles for DOT and CO2 on-line variables. Although similar on-line profile and off-line profile correlations were observed for defined and complex medium fermentations, off-line profile levels in complex medium fermentations are different due to the different metabolism of yeast cells grown on complex medium.

6.1.7 Mathematical programming techniques were used to base production scheduling on calculated parameters rather than predetermined schedules. Recognition of signals from the CO2 exit gas profiles is demonstrated. Two signals were identified from the CO2 profile of aerobic yeast culture fermentations on defined medium. One signal was distinguished from the CO2 profile of fermentations on complex medium. The LabVIEW
programming environment was enhanced to incorporate on-line process decision making for harvesting the intracellular enzyme products. Development of computer power and technology is incredibly fast whereas there is yet so much unknown in the field of physiology.

6.1.8 The off-line culture data (glucose, ethanol, biomass, protein, selected enzymes) and the on-line culture data (CO₂ exit gas content) for single batch and repeated batch aerobic culture of a Baker's yeast presented and discussed in chapter 3 have shown some surprising and controversial results.

i) The results were reproducible and gave parameter changes greater than the experimental error variance. Hence, the results changes were valid and significant (Appendices 2-5, and Chapter 3 comparison graphs).

ii) The strain of *S. cerevisiae* used for the fermentations in this project excreted pyruvate into the culture environment on exhaustion of the glucose supply in that culture environment, around 24 hours batch culture time for SBD4 (Figure 3.2-b). The pyruvate was then respired, presumably via the TCA cycle, and was consumed by the cells from the culture environment. The regulation of pyruvate transport from the membrane structure associated with the yeast cell mitochondria to the cell membrane and across the membrane to the liquid culture environment is unknown. The reversal of this remarkable leakage is also unknown, particularly with the sheer time scale involved. There is therefore unlikely to be any induction time for enzymes and or protein carrier system formation associated with this pyruvate leakage.

iii) The TCA enzymes (MDH) appear to be repressed for the first growth period (Appendix 2.2, Figure A2.2-d) up to the first CO₂ exit gas peak at 20 hours. In conjunction with other data in Figure A2.2, and Figure 6.1, the comparison of enzyme production with yeast cell biomass concentration presented in Figure 3.3, in particular the consistent yield of hexokinase and G6PDH in contrast with the significant changes in the yield of ADH and MDH in Figure 3.3 and that the respiratory quotient greater than unity (respiration of glucose) from 10 to 20 hours, indicated that the yeast cells only minimally respire glucose during phase (1), and that the fermentation was the main energy generation-process for Baker's yeast during exponential growth on glucose. This cell functioning was irrespective of the dissolved oxygen supply in the liquid culture environment, such that fermentation occurred when the dissolved oxygen content of the culture liquid medium was always greater than 60% saturation. This finding has a major impact on commercial yeast production via a sugar fed batch process and the understanding of the biotechnology of the process, for example should Baker's yeast be
aerobically grown on a mixture of sucrose and ethanol, provided the Distillers Baker's yeast used in this work is typical of *S. cerevisiae* Baker's yeast.

Figure 6.1 Carbon dioxide content (Vol%) in exit gas from aerobic batch bioreactor with Baker's yeast growth on glucose, experiment SBD2, and respiratory quotient (RQ), for the same batch culture.
iv) The respiration of the MDH and ADH enzymes during the so called observed aerobic growth on glucose poses the question as to whether this particular yeast has a limited respiratory capacity at all, or whether this yeast exhibits a true diauxic growth system for subsequent aerobic growth (respiration) on the ethanol produced and accumulated in the liquid culture environment during fermentation of the glucose in a 60% saturation dissolved oxygen culture liquor environment. As proposed in this thesis, limited respiratory capacity in yeasts could be variable, depending on the yeast type, cell culture history, actual culture environment and cell activity level. Further, the switching off and on (repression and derepression) of the enzyme production associated with energy generation and the mitochondria in yeast in comparison with enzyme production associated with glycolysis and general cellular function implies that the functioning of the mitochondria at least in yeast be reviewed as an organelle that can operate on an independent basis and is more susceptible to extra cellular environmental condition than has been considered to date in literature.

v) The RQ data in Figure 6.1, although with considerable noise in the data (for as long as 20 hours), supports these observations on yeast cells performance in aerobic batch culture. Pure respiration of glucose (RQ=1.0) did not occur during the first growth phase up to the first CO$_2$ exit gas peak. Subsequently, ethanol respiration (RQ=0.69) dominated although organic acid utilisation is considered by previous researchers to be occurring in the cell during the second and additional CO$_2$ exit gas peaks. The second growth phase of the yeast (diauxic growth after an induction lag) on ethanol substrate with respiration of the ethanol also raises the concept and implication of neoglucogenesis. In the yeast, as an explanation of the diauxic lag, and the exit gas analysis as an on-line measurement of the key cell function parameters of O$_2$ used and CO$_2$ production provides an excellent research tool for investigating such metabolic shifts.

6.1.9 On-line and off-line correlations showed good reproducibility in phases 1 to 4 of the CO$_2$ exit gas profiles for fermentations with defined medium. Reproducible correlations indicated that the process decision making of harvesting intracellular enzymes can be based on the CO$_2$ exit gas profiles. Harvesting time for a particular enzyme product depends entirely on the metabolism and intracellular enzyme activity levels. For example, yeast cells living on glucose are more efficient in producing ADH than those growing on ethanol. If the required enzyme activity level is that obtained by growth on glucose, the first peak of the CO$_2$ profile (phase 2) can then be chosen as the signal to identify the timing of the harvest. For a higher activity level of ADH, biomass must be harvested at the end of growth on ethanol which is indicated by the signal from the last peak of the CO$_2$ profile (phase 7). For MDH and hexokinase, the signal from the last peak (phase 7) of the CO$_2$ profile must be used for harvesting the cells. Optimum
harvesting time of the cells for G6PDH product is at phase 5 so that the signal from the first peak of the CO\(_2\) profile can be used with fermentation time for harvesting time of the cells (e.g. 14 hours after the start of phase 2 of the CO\(_2\) profile).

6.1.10 Consequently the objective of this research:

To use easily monitored variables (CO\(_2\) and DOT profiles) and the correlations of these variables with the biological events inside the batch bioreactor (nutrient depletion, and product formation) to aid process decision making (e.g. the timing of seed transfer, prediction of maximum product formation) for a better process operation and control of the batch bioreactor.

has been achieved, by completing the following aims:

a) Investigating the enzyme production levels during the exponential growth phase, through the transition into the stationary phase of the yeast growth. Intracellular enzymes such as G6PDH, hexokinase, ADH, and MDH were chosen as metabolic indicators of the transition phase.

b) Identifying the correlations between the on-line monitored variables and the intracellular enzyme activity levels measured off-line, and to indicate the reproducibility of the correlations between these variables.

c) Studying the reproducibility of the assay profiles and to reduce the batch to batch variability of the profiles by performing repeated batch cultures under the same fermentation conditions as the batch cultures.

d) Assessing the variability of the off-line measurements to obtain a knowledge of the measurement error for establishing the validity of the correlations and events observed in the fermentations.

e) Using mathematical techniques to identify a signal from the on-line monitored variables, indicating the transition of the fermentation phase, where process decisions such as timing of the harvest or refilling the tank could be based on the recognised signal.

Such that, The on-line variable measurement of inlet and exit gas composition, in particular the exit gas CO\(_2\) content provides not only a powerful tool for investigating the metabolic functioning of microbial cells in batch and continuous culture bioreactors, providing a
"finger print of cellular activity" with cellular environment and culture time, but also a sensitive, reliable and reproducible means of controlling and operating batch bioreactors for required products, as both single batch and repeated batch (drain and fill) fermentations. The on-line variable of $O_2/CO_2$ content of inlet/outlet gas streams should therefore enhance the industrial development of fully automated batch (unsteady) bioreactors.

6.2 Recommendations

With reference to the work presented and the conclusions made in section 6.1, the following recommendations are made:

6.2.1 The reproducibility of SBD4 and RBC1-2 fermentations must be examined, using a greater working volume for the fermentations to avoid large volume changes during broth sampling. Agitation is reported to have an affect on the length of the $CO_2$ profile (Locher et al, 1993). The effect of agitation on the $CO_2$ profile, and intracellular enzyme product formation also needs to be investigated in more detail for a better process optimisation.

6.2.2 The repeated batch culture technique can be used, by self-inoculating the fermenter at different phases of the $CO_2$ profile, to investigate the effect of inoculum size on the on-line $CO_2$ exit gas analysis profile. In such an experiment one could inoculate the last fermentation at the same time as the second batch of the experiment to check the possibility of any changes in the strain of the micro-organism used.

6.2.3 Glycerol measurement gives more information on the utilisation of this carbon source and correlation with $CO_2$ profile. Both glycerol and acetic acid can be measured with a HPLC. Turner (1993) developed a method for on-line monitoring of acetic acid and reported good reproducibility of up to 68 sequential samples with the on-line technique. In the work presented in this thesis, although analysis of glucose did not indicate great variabilities, ethanol analysis showed large variabilities. The most likely explanation for this observation is freezing the filtered samples from the fermentation broth and keeping the samples frozen for two to three months before defrosting for HPLC analysis. Experiments measuring ethanol content of a fresh sample and making a comparison with the same samples frozen for a period of time could determine whether...
the variability was due to processing the samples or if the change was due to variation in ethanol concentration in the broth.

The confirmation of the existence of acetic acid production during the aerobic growth of yeast on glucose and then ethanol should be confirmed as ethyl acetate production by yeast during aerobic growth on ethanol has been reported for some brewing yeasts.

6.2.4 Further in investigation of yeast metabolism in aerobic culture, both batch and continuous, for this Distillers strain of Baker's yeast should be made with particular reference to the existence of limited respiratory capacity and or diauxic activity with the possible repression-derepression of intracellular enzymes involved with cellular energy generation.

6.2.5 In order to confirm the success of using the CO$_2$ content of the exit gas from a bioreactor to characterise and thereby control the operation of a batch fermentation process, the CO$_2$ exit gas and RQ of other yeast types should be obtained. Initially with other strains of *S.cerevisiae* Brewer's and Baker's yeast, in aerobic batch culture to ascertain whether the metabolic performance of these yeasts is equivalent to the unexpected cellular performance of the Distillers Baker's yeast, (excretion of pyruvate, an extreme limited respiratory capacity, etc.). Then the yeasts should be cultured anaerobically to establish the correlation between CO$_2$ production and cellular functions under an anaerobic environment. Finally, aerobic batch cultures of the typical bacterium, eg *E.coli* and mycelium mold, eg. *Penicilium* should be carried out to establish whether the methodology and protocol of the CO$_2$ exit gas, as an on-line measurement, remains independent of the microbe substrate system and is therefore of general application to the fermentation industry.

6.2.6 Process scale up and investigation of the profiles and the correlation with metabolic product activity is also recommended. Use LabVIEW as a supervisory control for on-line execution of the product schedules where control sequence (emptying and refilling the fermenter) is initiated by the control parameter (signals from CO$_2$ exit gas analysis). Individual operation would be carried out automatically by the control sequence.
APPENDIX 1.

Calibrating the Turbidimetric Method For Baker's Yeast Biomass Determination:

Introduction:
Different methods of determining microbial concentration have been investigated by many authors (Sonnleitner et al., 1992). A simple method which gives a rapid determination of the off line microbial cell concentration is the turbidimetric method. The method is based on the Beer-Lambert empirical law, which defines the attenuation in the intensity of a light beam taken on the Pye Unicam single beam SP6-550 UV/VIS spectrophotometer at 660nm as absorbance.

Theory:
If a light of intensity \(I_0\) passes through a substance (which may be a solution) of thickness \(d\) and molar concentration \(c\), the intensity of transmitted light obeys the Beer-Lambert law.

\[
I = I_0 e^{-\varepsilon dc}
\]

or

\[
\log_{10}\left(\frac{I}{I_0}\right) = K_a c = -\varepsilon dc
\]

where \(\varepsilon\) = the molar extinction coefficient \([\text{cm}^2 \cdot \mu\text{mol}^{-1}]\)

For \(d=1\ [\text{cm}]\)

\[
\text{optical density} = \log_{10}\left(\frac{I}{I_0}\right) = K_a c
\]

Where \(\frac{I}{I_0}\) is the attenuation of light intensity

\(c\) is the concentration of all particles within the sample \([\text{mol} \cdot \text{L}^{-1}]\)

\(K_a\) is the calibration constant \([\text{cm}^3 \cdot \mu\text{mol}^{-1}]\)

The calibration constant \(K_a\) can be based reasonably on some cell mass function like dry cell weight, since cell suspension settling, flocculation, cell size and viability together with changes in culture medium affect the absorbance.
**Method:**

In the experiment 0.5 grams of baker's yeast were suspended in half a litre of a solution consisting the main salts of DW medium [Locher *et al.*, 1991].

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄</td>
<td>17.8</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>5.7</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>1.2</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>0.15</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.243</td>
</tr>
<tr>
<td>meso-Inositol</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table A1.1 The composition of the solution made (g/L) was as follows:

In order to construct a calibration curve of wet cell weight concentration against the absorbance, 0.5g of the yeast was suspended in 500ml of the DW solution. The 1g/L yeast suspension was then continuously stirred by gentle agitation of a magnetic stirrer. Additional DW medium was then used to make a series of dilutions of known yeast cell concentrations. The measurements were repeated 4 times with this solution. Absorbance of these diluted suspensions was taken on the Pye Unicam single beam SP6-550 spectrophotometer at 660nm, the optimum wavelength for yeast suspension determined by Knights (1981). Water was then used as the suspension media to study the difference between diluting the biomass with the media or pure water.

The wet mass concentration data were converted by the dry weight / wet weight ratio of 0.318, into the dry cell mass concentration data set. (This ratio was obtained by measuring one gram of the wet yeast and reweighing the same piece of yeast after drying in the oven again.)

The data set is shown in Table A1.2, and plotted in figures A1.1 to A1.3. In experiments 6-7 dilutions were made using distilled water (W), for the remaining experiments, samples were diluted using solution (S) from the main salts of the DW medium.
Table A1.2  Replicate measurements of optical density and dry cell weight concentration for several measurement sets. Both water (W) and major salts of the DW defined medium in a solution (S) were used to make up different concentration of the yeast suspension.

<table>
<thead>
<tr>
<th>Wet biomass</th>
<th>Dry biomass</th>
<th>OD S</th>
<th>OD S</th>
<th>OD S</th>
<th>OD S</th>
<th>OD S</th>
<th>OD W</th>
<th>OD W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. (g/L)</td>
<td>Conc. (g/L)</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
</tr>
<tr>
<td>Expt.no.</td>
<td>no. 1</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
</tr>
<tr>
<td>0.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>0.318</td>
<td>0.709</td>
<td>0.713</td>
<td>0.712</td>
<td>0.709</td>
<td>0.719</td>
<td>0.719</td>
<td>0.716</td>
</tr>
<tr>
<td>0.9</td>
<td>0.286</td>
<td>0.658</td>
<td>0.656</td>
<td>0.653</td>
<td>0.661</td>
<td>0.661</td>
<td>0.664</td>
<td>0.657</td>
</tr>
<tr>
<td>0.8</td>
<td>0.254</td>
<td>0.601</td>
<td>0.600</td>
<td>0.602</td>
<td>0.605</td>
<td>0.607</td>
<td>0.607</td>
<td>0.600</td>
</tr>
<tr>
<td>0.7</td>
<td>0.223</td>
<td>0.543</td>
<td>0.539</td>
<td>0.542</td>
<td>0.543</td>
<td>0.549</td>
<td>0.549</td>
<td>0.541</td>
</tr>
<tr>
<td>0.6</td>
<td>0.191</td>
<td>0.478</td>
<td>0.473</td>
<td>0.475</td>
<td>0.481</td>
<td>0.481</td>
<td>0.479</td>
<td>0.476</td>
</tr>
<tr>
<td>0.5</td>
<td>0.159</td>
<td>0.409</td>
<td>0.409</td>
<td>0.406</td>
<td>0.413</td>
<td>0.413</td>
<td>0.409</td>
<td>0.406</td>
</tr>
<tr>
<td>0.4</td>
<td>0.127</td>
<td>0.337</td>
<td>0.336</td>
<td>0.335</td>
<td>0.339</td>
<td>0.341</td>
<td>0.334</td>
<td>0.335</td>
</tr>
<tr>
<td>0.3</td>
<td>0.095</td>
<td>0.256</td>
<td>0.258</td>
<td>0.256</td>
<td>0.256</td>
<td>0.257</td>
<td>0.259</td>
<td>0.260</td>
</tr>
<tr>
<td>0.2</td>
<td>0.064</td>
<td>0.172</td>
<td>0.174</td>
<td>0.168</td>
<td>0.177</td>
<td>0.177</td>
<td>0.175</td>
<td>0.179</td>
</tr>
<tr>
<td>0.1</td>
<td>0.032</td>
<td>0.093</td>
<td>0.089</td>
<td>0.089</td>
<td>0.092</td>
<td>0.092</td>
<td>0.088</td>
<td>0.092</td>
</tr>
</tbody>
</table>

Table A1.3  At concentrations 0.3 g/L and 0.8 g/L, 5 more replicates of the measurements were performed using solution S to suspend the yeast.

<table>
<thead>
<tr>
<th>Wet biomass</th>
<th>Dry biomass</th>
<th>OD S</th>
<th>OD S</th>
<th>OD S</th>
<th>OD S</th>
<th>OD S</th>
<th>OD S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. (g/L)</td>
<td>Conc. (g/L)</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
</tr>
<tr>
<td>Expt.no.</td>
<td>no. 8</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
<td>660</td>
</tr>
<tr>
<td>0.3</td>
<td>0.095</td>
<td>0.263</td>
<td>0.261</td>
<td>0.259</td>
<td>0.258</td>
<td>0.260</td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>0.254</td>
<td>0.603</td>
<td>0.608</td>
<td>0.608</td>
<td>0.606</td>
<td>0.606</td>
<td></td>
</tr>
</tbody>
</table>
Figure A1.1  Calibration curve of absorbance verses yeast cell concentration. In experiments 1-5 DW medium is used for dilutions whereas in experiment 6 and 7 water is used for dilutions. The results show a linear response for absorbance values of below 0.3.

Figure A1.2  Calibration curve of absorbance verses yeast cell concentration. All samples are diluted using DW medium. Dilutions are repeated five times for each sample.
Figure A1.3  Calibration curve of absorbance verses yeast cell concentration. All samples are diluted using water. Dilutions are repeated twice for each sample.

Samples from the fermenter must be diluted with either the salt solution or water to reduce the biomass suspension in the original fermenter liquid to a concentration that is within the linear region of the calibration graphs shown in Figures A1.1, A1.2 and A1.3. Therefore the absorbance reading of the spectrophotometer should be kept less than 0.3 at 660 nm.
APPENDIX 2

Experimental data for single batch cultures of *S. cerevisiae* using defined medium 'SBD'.

A total of four single batch cultures were carried out at a temperature of 28 °C and pH 4.5 and inlet air flow rate of 2.5 L/min, using defined medium with initial glucose concentration of 30 g/L as the limiting substrate.

On-line CO$_2$ exit gas data and DOT data were monitored from inoculation time to the end of growth on ethanol for all batches. Data presented in this section are from phase 1 to phase 4 of the CO$_2$ exit gas profile. Off-line fermentation broth was analysed for biomass, glucose, and ethanol concentrations and the activity levels of some intracellular enzymes (ADH, MDH, G6PDH, and hexokinase) as described in chapter 2.

Appendix 2 includes the basic experimental results for 3 single batches on defined medium (SBD1, SBD2, and SBD3). Inoculum to all batches were 250 mL of the culture grown on defined medium in a shake flask.

Experimental results for SBD1 are given in appendix 2.1.

Experimental results for SBD2 are given in appendix 2.2.

Experimental results for SBD3 are given in appendix 2.3.
Appendix 2.1

Figure A2.1-a CO₂ exit gas profile - ■ - and DOT profile - - - of single batch culture 'SBD1' on define medium.

Figure A2.1-b Off-line data of glucose - • - and ethanol, - ▲ - profile in single batch SBD1

Figure A2.1-c Dry cell weight concentration - □ - and total protein content - ■ - analysis of single batch fermentation SBD1 in define medium.
Figure A2.1-f  Off-line assay of Alcohol dehydrogenase - △ - (U/ml) and Malate dehydrogenase - ▼ - (U/ml) in single batch fermentation.

Figure A2.1-g  Glucose-6-phosphate dehydrogenase activity levels -▼ - (U/mL) in single batch fermentation SBD1.
Appendix 2.2

Fermentation SBD2

Figure A2.2-a  CO₂ exit gas profile - ■ - and DOT profile - - - - of single batch culture 'SBD2' on define medium.

Figure A2.2-b  Off-line data of glucose - ● -, ethanol, - ▲ - profile in single batch SBD2 with defined medium.

Figure A2.2-c  Dry cell weight concentration - □ - and total protein content - ■ - analysis of single batch fermentation SBD2 in defined medium.
Appendix 2.2 Fermentation SBD2

Figure A2.2-d Off-line assay of Alcohol dehydrogenase (U/mL) - △ - and Malate dehydrogenase (U/mL) - ▼ - in single batch fermentation.

Figure A2.2-e Glucose-6-phosphate dehydrogenase activity levels (U/mL) - ▼ - in single batch fermentation SBD2.
Appendix 2.3  

**Fermentation SBD3**

Figure A2.3-a  CO₂ exit gas profile - ■ - and DOT profile - - - of single batch culture 'SBD3' on defined medium.

Figure A2.3-b  Off-line data of glucose - • - ethanol, - ▲ - profile in single batch SBD3 with defined medium.

Figure A2.3-c  Dry cell weight concentration - □ - and total protein content - ■ - analysis of single batch fermentation SBD3 in defined medium.

213
Figure A2.3-d  Off-line assay of Alcohol dehydrogenase - △ - and MDH activity levels (U/mL) - ▼ - in single batch fermentation.

Figure A2.3-e  Glucose-6-phosphate dehydrogenase activity levels (U/mL) - ▼ - and hexokinase enzyme activity level (U/mL) - △ - in single batch fermentation SBD3.
APPENDIX 3

Experimental data for a repeated batch culture of *S.cerevisiae* using defined medium 'RBD1'.

RBD1 is a repeated batch culture with a total of two single batch cultures at a temperature of 28 °C and pH 4.5 and inlet air flow rate of 2.5 L/min, using defined medium with initial glucose concentration of 30 g/L as limiting substrate.

On-line CO$_2$ exit gas data and DOT data were monitored from inoculation time to the end of growth on ethanol for all batches. Data presented for RBD1-1 is from phase 3 to phase 7 of the CO$_2$ exit gas profile. RBD1-2, however, is analysed from phase 1 to phase 4. Off-line fermentation broth was analysed for biomass, glucose, and ethanol concentrations and the activity levels of some intracellular enzymes (ADH, MDH, G6PDH, and hexokinase) as described in chapter 2.

Appendix 3 includes the basic experimental results for RBD1 repeated batch culture on defined medium (RBD1-1, and RBD1-2). Inoculum to RBD1-1 was 250 mL of the culture from shake flask grown on defined medium. RBD1-2 was started with 1 litre of remaining biomass from RBD1-1.

Experimental results for RBD1-1 are given in appendix 3.1.

Experimental results for RBD1-2 are given in appendix 3.2.
Figure A3.1-a  CO₂ exit gas profile - x - and DOT profile - o - of repeated batch culture 'RBD1-1' on defined medium.

Figure A3.1-b  Off-line data of glucose - ● - , and ethanol - ▲ - profile in repeated batch RBD1-1 with defined medium.
Appendix 3.1. Fermentation RBD1-1

Figure A3.1-c  Dry cell weight concentration - □ - and total soluble protein content - ■ - analysis of repeated batch fermentation RBD1-1 in defined medium.

Figure A3.1-d  Off-line assay of Alcohol dehydrogenase - △ - (U/mL) and Malate dehydrogenase - ▼ - (U/mL) in repeated batch fermentation RBD1-1.

Figure A3.1-e  Glucose-6-phosphate dehydrogenase activity levels (U/mL) - ▼ - in repeated batch fermentation RBD1-1.
Appendix 3.2. 

Fermentation RBD1-2

Figure A3.2-a CO₂ exit gas profile of repeated batch culture 'RBD1-2' on defined medium.

Figure A3.2-b Off-line data of glucose - ● - , and ethanol - ▲ - profile in repeated batch RBD1-2 with defined medium.

Figure A3.2-c Dry cell weight concentration - □ - and total soluble protein content - ■ - analysis of repeated batch fermentation RBD1-2 in defined medium.
Appendix 3.2. Fermentation RBD1-2

Figure A3.2-d  Off-line assay of Alcohol dehydrogenase - △ - (U/mL) and Malate dehydrogenase - ▼ - (U/mL) in repeated batch fermentation RBD1-2.

Figure A3.2-e  Glucose-6-phosphate dehydrogenase activity levels (U/mL) - ▼ - in repeated batch fermentation RBD1-2.
APPENDIX 4

Experimental data for a repeated batch culture of *S. cerevisiae* using defined medium 'RBD2'.

RBD2 is a repeated batch culture with a total of three single batch cultures at a temperature of 28 °C and pH 4.5 and inlet air flow rate of 2.5 L/min, using defined medium with initial glucose concentration of 30 g/L as limiting substrate.

On-line DOT data were monitored from inoculation time to the end of growth on ethanol for all batches. CO₂ exit gas data are not available for this fermentation due to mass spectrometer failure. Data presented for individual batches are from phase 1 to phase 4 of the CO₂ exit gas profile. Off-line fermentation broth was analysed for biomass, glucose, and ethanol concentrations and the activity levels of some intracellular enzymes (ADH, MDH, G6PDH, and hexokinase) as described in chapter 2.

Appendix 4 includes the basic experimental results for RBD2 repeated batch culture on defined medium (RBD2-1, RBD2-2, and RBD2-3). Inoculum to RBD2-1 was 250 mL of the culture from shake flask grown on defined medium. Following individual batches started by using 250 mL of the culture from the previous batch.

Experimental results for RBD2-1 are given in appendix 4.1.

Experimental results for RBD2-2 are given in appendix 4.2.

Experimental results for RBD2-3 are given in appendix 4.3.
Figure A4.1-a DOT profile - - - of repeated batch culture 'RBD2-1' on defined medium.

Figure A4.1-b Off-line data of glucose - ■ -, ethanol - ▲ -, and pyruvate - ○ - profile in repeated batch RBD2-1 with defined medium.

Figure A4.1-c Dry cell weight concentration - □ - and total soluble protein content - ■ - analysis of repeated batch fermentation RBD2-1 in defined medium.
Appendix 4.1. Fermentation RBD2-1

Figure A4.1-d Off-line assay of Alcohol dehydrogenase - △ - (U/mL) and Malate dehydrogenase - ▽ - (U/mL) in repeated batch fermentation.

Figure A4.1-e Glucose-6-phosphate dehydrogenase activity levels (U/mL) - ▽ - and Hexokinase activity levels (U/mL) - ◊ - in repeated batch fermentation RBD2-1.
Appendix 4.2.

Fermentation RBD2-2

Figure A4.2-a  DOT profile - - - of repeated batch culture 'RBD2-2' on defined medium.

Figure A4.2-b  Off-line data of glucose - , ethanol - , and pyruvate - profile in repeated batch RBD2-2 with defined medium.

Figure A4.2-c  Dry cell weight concentration - and total soluble protein content - analysis of repeated batch fermentation RBD2-2 in defined medium.
Appendix 4.2. Fermentation RBD2-2

Figure A4.2-d Off-line assay of Alcohol dehydrogenase - Δ - (U/mL) and Malate dehydrogenase - ▼ - (U/mL) in repeated batch fermentation.

Figure A4.2-e Glucose-6-phosphate dehydrogenase activity levels (U/mL) - ▼ - and Hexokinase activity levels (U/mL) - ◆ - in repeated batch fermentation RBD2-2.
Appendix 4.3. Fermentation RBD2-3

Figure A4.3-a DOT profile - of repeated batch culture 'RBD2-3' on defined medium.

Figure A4.3-b Off-line data of glucose - , ethanol - , and pyruvate - profile in repeated batch RBD2-3 with defined medium.

Figure A4.3-c Dry cell weight concentration - and total soluble protein content - analysis of repeated batch fermentation RBD2-3 in defined medium.
Appendix 4.3. Fermentation RBD2-3

Figure A4.3-d Off-line assay of Alcohol dehydrogenase - △ - (U/mL) and Malate dehydrogenase - ▼ - (U/mL) in repeated batch fermentation.

Figure A4.3-e Glucose-6-phosphate dehydrogenase activity levels (U/mL) - ▼ - and Hexokinase activity levels (U/mL) - ◊ - in repeated batch fermentation RBD2-3.
APPENDIX 5

Experimental data for a repeated batch culture of *S. cerevisiae* using defined medium 'RBD3'.

RBD3 is a repeated batch culture with a total of four single batch cultures at a temperature of 28 °C and pH 4.5 and inlet air flow rate of 2.5 L/min, using defined medium with initial glucose concentration of 30 g/L as limiting substrate. First batch of this experiment (RBD3-1) was discarded, since the data acquisition had failed overnight and on-line data were not available before off-line analysis of the fermentation broth.

On-line CO$_2$ exit gas data and DOT data were monitored from inoculation time to the end of growth on ethanol for all batches. CO$_2$ exit gas data shown in this section are normalised as described in section 3.2.4, since mass spectrometer was out of calibration for the first two batches. Data presented for individual batches are from phase 1 to phase 4 of the CO$_2$ exit gas profile. Off-line fermentation broth was analysed for biomass, glucose, and ethanol concentrations and the activity levels of some intracellular enzymes (ADH, MDH, G6PDH, and hexokinase) as described in chapter 2.

Appendix 5 includes the basic experimental results for RBD3 repeated batch culture on defined medium (RBD3-3, RBD3-4, and RBD3-4). Inoculum to each batch was 500 mL of the culture from the previous batch.

Experimental results for RBD3-1 are given in appendix 5.1.

Experimental results for RBD3-2 are given in appendix 5.2.

Experimental results for RBD3-3 are given in appendix 5.3.
Appendix 5.1. Fermentation RBD3-2

Figure A5.1-a CO₂ exit gas profile - - - and DOT profile - - - of repeated batch culture 'RBD3-2' on defined medium.

Figure A5.1-b Off-line data of glucose - ● -, ethanol - ▲ -, and pyruvate - ○ - profile in repeated batch RBD3-2 with defined medium.

Figure A5.1-c Dry cell weight concentration - ◻ - and total soluble protein content - ■ - analysis of repeated batch fermentation RBD3-2 in defined medium.
Appendix 5.1. Fermentation RBD3-2

Figure A5.1-d  Off-line assay of Alcohol dehydrogenase - △ - (U/mL) and Malate dehydrogenase - ▼ - (U/mL) in repeated batch fermentation.

Figure A5.1-e  Glucose-6-phosphate dehydrogenase activity levels (U/mL) - ▼ - and Hexokinase activity levels (U/mL) - ◊ - in repeated batch fermentation RBD3-2.
Appendix 5.2. Fermentation RBD3-3

Figure A5.2-a  CO₂ exit gas profile - - - and DOT profile - - - of repeated batch culture 'RBD3-3' on defined medium.

Figure A5.2-b  Off-line data of glucose - • -, ethanol - ▲ -, and pyruvate - ○ - profile in repeated batch RBD3-3 with defined medium.

Figure A5.2-c  Dry cell weight concentration - □ - and total soluble protein content - ■ - analysis of repeated batch fermentation RBD3-3 in defined medium.
Appendix 5.2. Fermentation RBD3-3

Figure A5.2-d Off-line assay of Alcohol dehydrogenase - Δ - (U/mL) and Malate dehydrogenase - ▼ - (U/mL) in repeated batch fermentation.

Figure A5.2-e Glucose-6-phosphate dehydrogenase activity levels (U/mL) - ▼ - and Hexokinase activity levels (U/mL) - ◊ - in repeated batch fermentation RBD3-3.
Appendix 5.3. Fermentation RBD3-4

Figure A5.3-a CO2 exit gas profile - - - and DOT profile - - - of repeated batch culture 'RBD3-4' on defined medium.

Figure A5.3-b Off-line data of glucose - , ethanol - ▲ - , and pyruvate - ○ - profile in repeated batch RBD3-4 with defined medium.

Figure A5.3-c Dry cell weight concentration - □ - and total protein content - ■ - analysis of repeated batch fermentation RBD3-4 in defined medium.
Appendix 5.3.  

Fermentation RBD3-4

Figure A5.3-d  
Off-line assay of Alcohol dehydrogenase - △ - (U/mL) and Malate dehydrogenase - ▼ - (U/mL) in repeated batch fermentation RBD3-4.

Figure A5.3-e  
Glucose-6-phosphate dehydrogenase activity levels (U/mL) - ▼ - and Hexokinase activity levels (U/mL) - ○ - in repeated batch fermentation RBD3-4.
APPENDIX 6

Experimental data for an individual batch culture of *S. cerevisiae* using complex medium from repeated batch culture 'RBC2'.

RBC2 is a repeated batch culture with a total of four single batch cultures at a temperature of 28 °C and pH 4.5 and inlet air flow rate of 2.5 L/min, using complex medium with initial glucose concentration of 30 g/L as limiting substrate. First batch of this experiment (RBD3-1) was discarded, since the data acquisition had failed overnight and on-line data was not available before off-line analysis of the fermentation broth. This experiment was designed to investigate the reproducibility of the on-line data from complex medium fermentations (Figure 3.10-a,b).

On-line CO₂ exit gas data and DOT data were monitored from inoculation time to the end of growth on ethanol for all batches. Data presented for RBC2-2 are from phase 1 to phase 4 of the CO₂ exit gas profile. Off-line fermentation broth was only analysed for RBC2-2. Biomass, glucose, and ethanol concentrations and the activity levels of some intracellular enzymes (ADH, MDH, G6PDH, and hexokinase) were analysed as described in chapter 2.

Appendix 6 includes the basic experimental results for RBC2-2 repeated batch culture on complex medium. RBC2-1 was inoculated from 250 ml of the culture grown on complex medium in a shake flask. Inoculum to the following batches were 1 litre of the culture from the previous batch.

Experimental results for RBC2-2 are given in appendix 6.1.
Appendix 6.1. Fermentation RBC2-2

Figure A6.2-a  CO₂ exit gas profile - • - and DOT profile - - - of repeated batch culture 'RBC2-2' on complex medium.

Figure A6.2-b  Off-line data of glucose - • -, ethanol - ▲ - and pyruvate - ○ - profile in repeated batch RBC2-2 with complex medium.

Figure A6.2-c  Dry cell weight concentration - □ - and total soluble protein content - ■ - analysis of repeated batch fermentation RBC2-2 in complex medium.
Figure A6.2-d Off-line assay of Alcohol dehydrogenase - △ - (U/mL) and Malate dehydrogenase - ▽ - (U/mL) in repeated batch fermentation RBC2-2.

Figure A6.2-e Glucose-6-phosphate dehydrogenase activity levels (U/mL) - ▽ - and hexokinase enzyme activity level (U/mL) - ◊ - in single batch fermentation RBC2-2.
APPENDIX 7.1

LabVIEW visual program code to get CO$_2$ data.

Figure A7.1 LabVIEW visual program code to get CO$_2$ data.
APPENDIX 7.2

LabVIEW visual program code to detect the first and the last CO₂ peaks.

Figure A7.2  LabVIEW visual program code to detect the first and the last CO₂ peaks.
REFERENCES

ABEL C., HUBNER U., SCHUGERL K.
"Transient behaviour of Baker's yeast during enforced periodical variation of dissolved oxygen concentration."
*Journal of Biotechnology.*

AHLMAN N., NIEHOFF A., RINAS U., SCHEPER Th., and SCHUGERL K.
"Continuous monitoring of intracellular enzyme activity."
*Analytica Chemica Acta.*
190, 221-226, (1986).

AHMAD M. N., HOLLAND C. R., MCKAY G.
"Growth characteristic of candida utilis in aerobic batch fermentation."
THE 1991 ICHME RESEARCH EVENT.
Department of Chemical Engineering.
Research and Technology Symposium. Cambridge.

AIBA S., SHODA M., NAGATANI M.
"Kinetics of product inhibition in alcohol fermentation."
*Biotech. Bioeng.*

ALEXANDER M. A. and JEFFRIES, T.W.
"Respiratory efficiency and metabolic partitioning as regulatory phenomena in yeasts."
*Enzyme Microb. Technol.*

ANDREYEVA L. N., BIRYUKOV V. V., SIKTYA B.
"Analysis of the mathematical models of the effect of pH on fermentation processes and their use for calculating optimal fermentation conditions."

ARMIGER W. B.
"Instrumentation for monitoring and controlling bioreactors."
Comprehensive Biotechnology.
' The principles of biotechnology: Science fundamental.'
Ed. Murray Moo-Young. Pergamon press Ltd.
2, 133-147, (1985).

ATKINSON B., MAVITUNA F.
"Biochemical engineering and biotechnology handbook."

AUBERSON L. C. M., STOCKER U. V.
"A unified stochiometric model for oxidative and oxidoreductive growth of yeast."
*Biotechnology and Bioengineering.*

"Large scale production of monoclonal antibodies in suspention culture."
*Biotech. Bioeng.*
32, 993-1000, (1988)
BAILEY J. E., OLLIS D. F.
"Other environmental effects on growth kinetics."
Biochemical Engineering Fundamentals.

BARFORD J. P.
"A mathematical model for the aerobic growth of Saccharomyces cerevisiae with a saturated respiratory capacity."
XXIII, 1735-1762, (1981)

BARFORD J. P.
"The technology of aerobic yeast growth."
Yeast Biotechnology.
Edtd. Berry D. R., Russell I., and Stewart G. G.

BARFORD J. P.
"A general model for aerobic yeast growth: Batch Growth."
Biotechnology and Bioengineering.

BARFORD J. P.
"A general model for aerobic yeast growth: Continuous Growth."
Biotechnology and Bioengineering.

BECK B., KASPAR von MEYENBURG H.
"Enzyme pattern and aerobic growth of Saccharomyces cerevisiae under various degrees of glucose limitation."
Journal of Bacteriology.

BELLGARDT K-H., YUAN J.
Volume 4, Measuring Modelling and Control.

BERGMAYER H. U.
"Methods of enzymic analysis."
Verlag Chemie, Weiheim, Germany.

BERRY D. R., BROWN C.
"Physiology of yeast growth."
Yeast Biotechnology.
Edt. Berry D. R., Russell I., Stewart G. G.

BLANKENSTEIN G., KULA M-R.
"Cell permeabilisation as a tool for measurement of intracellular enzyme activity in a flow-injection system."
Analytica Chimica Acta.
BOUCHER H.  
"Protein synthesis during transition and stationary phase under glucose limitation in Saccharomyces cerevisiae."  
*Journal of Bacteriology.*  

BRADFORD M. M.  
"A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye-binding."  
*Analytical Biochem.*  

BRADFORD J. P., HALL R. J.  
"An examination of the crabtree effect in S. cerevisiae."  
*Journal of General Microbiology.*  

BRADLEY J., STOCKLEIN W., SCHMID R. D.  
"Biochemistry based analysis systems for bioprocess monitoring and control."  
*Process Control and Quality.*  

BROOKS S. L., ASHBY R. E., TURNER A. P. F., CALDER M. R., and CLARKE D. J.  
"Development of an on-line glucose sensor for fermentation monitoring."  
*Biosensors.*  

BUCKLAND B. C.  
"Fermentation exhaust gas analysis using mass spectrometry."  
*Biotechnology.*  

BUCKLAND B. C.  
"The application of computer control to improve fermentation processes."  
Computer Control of Fermentation Processes.  
Edts. Omstead D., Raton B, Fla. CRC Press  

BULL D. N.  
"Instrumentation for fermentation process control."  
'The principles of biotechnology: Science fundamental.'  
Comprehensive Biotechnology.  
Edtd. Murray Moo-Young. Pergamon press Ltd.  

BURROWS S.  
"Baker's Yeast."  
The Yeast  
Edts. Rose A. H., and Harrison J. S.  

CAMELBEECK J. P., COMBERBACH D. M., GOOSSENS J., ROELANTS P.  
"On-line fermenter headspace gas analysis of methanol and ethanol by capillary inlet mass spectrometry."  
*Biotech. Techniques.*  
CARLYSMITH S. W., FOX R. I.  
"Fermentation instrumentation and control."  
*Adv. in Biotechnological Processes.*  

CARLYSMITH S. W.  
"Data handling for fermentation development."  
*IFAC,* Fourth International Congress on:  
Computer Applications in Fermentation Technology.  
Edited by ; N. M. Fish, R. I. Fox  

CHEN K-C., WU W-T., CHANG K-Y.  
"Computer control of continuous cultivation of *Saccharomyces cerevisiae.*"  

CHEN S. L., and CHIGER M.  
"Production of Baker's yeast."  
Comprehensive Biotechnology.  
'The principles of biotechnology: Science fundamental.'  
Edited. Murray Moo-Young. Pergamon press Ltd.  

CHEN Q., WEIGAND W. A.  
"Adaptive optimal operation of a bioreactor based on a neural net model."  
*IFAC* Modelling and Control of Biotechnical Process.  
Colorado, USA.  

CHERUY A., DURAND A.  
"Optimization of erythromycin biosynthesis by controlling pH and and temperature:  
Theoretical aspects and practical application."  

COPPELLA S. J., DHURJATI P.  
"A mathematical description of recombinent yeast."  
*Biotech. Bioeng.*  

COPPELLA S. J., DHURJATI P.  
"A detailed analysis of *Saccharomyces cerevisiae* growth kinetics in batch, fed-batch, and  
hollow fiber bioreactor."  
*The Chemical Engineering Journal.*  

COPPELLA S. J., DHURJATI P.  
"Low cost computer-coupled fermenter off-gas analysis via quadrupole mass spectrometer."  
*Biotechnology and Bioengineering*  

CORTASSA S., AON M. A.  
"Metabolic control analysis of glycolysis and branching to ethanol production in chemostat  
culture of *Saccharomyces cerevisiae* under carbon, nitrogen, or phosphate limitations.  
CRABTREE H. B.
"Observation on the carbohydrate metabolism of tumors."
*Biochem. J.*
23, 536-545, (1929).

DARLEY-USMAR V. M., RICKWOOD D., and WILSON M. T.
"Mitochondria: A practical approach."

DI MASSIMO-PEEL C., MONTAGUE G. A., WILLIS M. J., MORRIS A. J., THAM M. T.
"Enhanced industrial bioprocess monitoring through artificial neural networks."
*IFAC Modelling and Control of Biotechnical Process.*
Colorado, USA.

DINCER A. K., KALYANPOUR M., SKEA W., RYAN M., KIERSTEAD T.
"Continuous on line monitoring of fermentation processes."
Development in Industrial Microbiology.

ENDO H., SODE K., KARUBE I., MURAMATSU H.
"On-line monitoring of the viscosity in dextran fermentation using piezoelectric quartz crystal."
Biotechnology and Bioengineering.

FIECHTER A.
"Physical and chemical parameters of microbial growth."

FIECHTER A., AND VON MEYENBURG K.
"Regulatory properties of growing cell populations of *Saccharomyces cerevisiae* in a continuous culture system."
The Proceedings of the 2nd Int. Symposium on Yeast.
Bratislava.

FIECHTER A., FUHRMANN G. F., and KAPPELI O.
"Regulation of glucose metabolism in growing yeast cells."

FIECHTER A., KAPPELI O., and MEUSSDOERFFER F.
"Batch and continuous culture."
The Yeast. 'Yeast and Environment.'
Edited by Rose A.H., and Harrison J.S.

FIECHTER A., and SEGHEZZI W.
"Regulation of glucose metabolism in growing yeast cells."
*J Biotechnol.*
"Controlled expression and purification of human immune interferon from high cell density fermentations of Saccharomyces cerevisiae."
*Biotech. Bioeng.*

FLYNN D. S.
"Instrumentation for fermentation processes."
Modelling and Control of Biotechnological Processes.
*IFAC,* Helsinki
5-12, (1982).

FOWLER P. W., BALL A. J. S., GRIFFITHS D. E.
"The control of alcohol dehydrogenase isozyme synthesis in *S. cerevisiae.*"
*Canadian Journal of Biochemistry.*

FRAENKEL D. G.
"Carbohydrate metabolism."
The Molecular Biology of the Yeast, *S. cerevisiae.* Metabolism and Gene Expression.
Eds. Strathem J. N., Jones E. W., Broach J. R.

FUHRMANN G. F., VOLKER B.
"Regulation of glucose transport in *Saccharomyces cerevisiae.*"
*Journal of Biotechnology.*

GANCEDO C., SERRANO R.
"Energy yielding metabolism."
The Yeasts. Metabolism and Physiology of Yeast.

GBEWONYO K., JAIN D., HUNT G., DREW S. W., BUCKLAND B. C.
"On-line analysis of avermectin fermentation cell growth kinetics in an industrial pilot plant."
*Biotechnology and Bioengineering.*

GOWDA L. R., JOSHI M. S., BHAT S. G.
"*In situ* assay of intracellular enzymes of yeast (*Kluyveromyces fragilis*) by digitonin permeabilization of cell membrane."
*Analytical Biochemistry.*
175, 531-536, (1988).

GREGORY M.E., KEAY P.J., DEAN P, BULMER M, THORNHILL N.F.
"A visual programming environment for bioprocess control."
*Journal of biotechnology.*

GREGORY M.E, BULMER M., BOGLE I. D. L., TITCHENER-HOOKER N.
"A physiological study of enzyme productivity of bakers yeast grown in continuous culture."
(In preparation).
HALM Z.
"An advanced process manufacturing system - Design and application to a food processing pilot plant."
Imperial College of Science Technology and Medicine.
University of London.
chapter 7, 1995.

HALME A.
"Expert system approach to recognize the state of fermentation technology."
Modelling and Control of Biotechnological Processes.
IFAC, (Eds. N. M. Fish, R. I. Fox).

HALME A., VISALA A.
"Combining symbolic and numerical information in modelling the state of biotechnological processes."
International Symposium on Bioprocess Monitoring and Control.
Department of Chemical Engineering.
University of Newcastle.

HARRIS C., TODD R., BUNGARD S., LOVITT R., MORRIS G., KELL D.
"Dielectric permittivity of microbial suspensions at radio frequencies: a novel method for the realtime estimation of microbial biomass."

HEINZLE E.
"Mass spectrometry for on-line monitoring of biotechnological processes."

HEINZLE E.
"Present and optional applications of mass spectrometry for bioprocess research and control."
Journal of Biotechnology.
25, 81-114, (1992)

HEINZLE E., and DUNN E.
"Methods and instruments in fermentation gas analysis."
Biotechnology.
Volume 4, Measuring Modelling and Control.

HEINZLE E., OEGGERLI A., DETTWILER B.
"On-line fermentation gas analysis: error analysis and application of mass spectrometry."
Analytica Chimica Acta.

HOLZER H.
"Catabolite inactivation in yeast."
Trends in Biochemical Sciences
HORIUCHI J., KAMASAWA M., MIYAKAWA M., KISHIMOTO M.  
"On-line control system of fed-batch culture with culture phase recognition using fuzzy inference."  
IFAC Modelling and Control of Biotechnical Process.  
Colorado, USA.  

JALEL N. A., TSAPTSINOS D., MIRZAI A. R., LEIGH J. R., DIXON K.  
"Modelling the oxytetracycline fermentation process using multi-layered perceptrons."  
IFAC Modelling and Control of Biotechnical Process.  
Colorado, USA.  

JULIAN K. S.  
"Effect of yeast culture storage conditions on the kinetic performance of a brewing yeast in batch culture."  
The IChemE Research Event.  
Research and technology Symposium, Cambridge.  

KALLE G. P., NAIK S. C.  
"Effect of controlled aeration on glycerol production in a sulphite process by *Saccharomyces cerevisiae*."  
*Biotech. Bioeng.*  

KAPPELI O.  
"Regulation of carbon metabolism in yeast."  

KEMBLOWSKI Z., KRISTIANSEN B., AJAYI O.  
"On-line rheometer for fermentation liquids."  
*Biotechnology Letters*  

KNIGHTS A. J.  
"Determination of the biological kinetic parameters of fermenter design from batch culture data."  

KRACKE-HELM H-A, BRANDES L, HITZMANN B., RINAS U., SCHUGERL K.  
"On-line determination of intracellular β-glactosidase activity in recombinant *Escherichia coli* using flow injection analysis (FIA)."  
*Journal of Biotechnology.*  

LAFON-LAFOURCADE S., GENEIX C., and RIBEREAU-GAYON P.  
"Inhibition of alcoholic fermentation of grape must by fatty acids produced by yeast and their elimination by yeast ghosts."  
*Applied Environmental Microbiology.*  
LINTON J. D., STEPHENSON R. J.
"A preliminary study on growth yields in relation to the carbon and energy content of various organic growth substrates."
FEMS Microbiol. Lett.

LOCHER G., SONNLEITNER B., and FIECHTER A.
"Automatic Bioprocess Control. 3. Impacts on Process Perception."
J. Biotechnol.

LOCHER G., SONNLEITNER B., and FIECHTER A.
"A. On-Line Measurement in Biotechnology."
J. Biotechnol.

LOCHER G., HAHNEMANN U., SONNLEITNER B., and FIECHTER A.
"Automatic bioprocess control. 4. A prototype batch of Saccharomyces cerevisiae."
J. Biotechnol.

LOCHER G., SONNLEITNER B., and FIECHTER A.
"Automatic bioprocess control. 5. Biologically and technically caused effects during cultivation."
J. Biotechnol.

LOUREIRO V., and FERREIRA H. G.
"On the intracellular accumulation of ethanol in yeast."
Biotechnology and Bioengineering.

LUNDELL R.
"Practical implementation of basic computer control strategies for enzyme productions."
Computer applications in fermentation technology.
London, SCI.

MAITRA P.K., and LOBO Z.
"A kinetic study of glycolitic enzyme synthesis in yeast."
J. Biol. Chem.

MAITRA P.K., and LOBO Z.
"Control of glycolitic enzymes synthesis in yeast by products of the Hexokinase reaction."
J. Biol. Chem.

MAIORELLA B., BLANCH H. W., and WILKE C. R.
"By-product inhibition effects on ethanoic fermentation by Saccharomyces cerevisiae."
Biotechnology and Bioengineering.
MARSHALL C. T.
"The design and implementation of comparative reasoning tools for fermentations."
August, 1992.

MARSHALL C. T., THORNHILL N. F., LILLY M. D., BUCKLAND B. C., FISH N. M.
"Design and use of an automated fermentation analysis system utilising historical data."
IFAC Modelling and Control of Biotechnical Processes.

MAURICIO J. C., and ORTEGA J. M.
"Influence of fermentation conditions on specific activity of the enzymes alcohol and aldehyde dehydrogenase from yeasts."
Microbios.

McCANN E. P., CALAM C. T.
"The metabolism of Penicillin chrysogenum and the product of Penicillin using a high yielding strain at different temperature."

MONTAGUE G. A., MORRIS A. J., WARD A. C.
"Fermentation monitoring and control: A Perspective."
Biotechnology and Genetically Engineering Reviews.

MOU D. G., COONY C.
"Growth monitoring and control through computer aided on-line mass balancing in a fed-batch Penicillin fermentation."

NAGODAWITHANA T. W., STEINKRAUS H. K.
"Influence of the rate of ethanol production and accumulation on the viability of Saccharomyces cerevisiae in a rapid fermentation."
Applied Environmental Microbiology.

OAKLEY R. V.
"Apparatus and method for periodic aseptic withdrawal of liquid samples from a sterile liquid source."
Patent.

OMSTEAD D. R., PHILLIPS J. A., HUMPHREY A. E.
"Indirect parameter estimation."
Computer Control of Fermentation Processes.
Eds. Omshead D., Raton B, Fla. CRC Press

PAMPULHA M. E., LOUREIRO V.
"Interaction of the effects of acetic acid and ethanol on inhibition of fermentation in Saccharomyces cerevisiae."
Biotechnology Letters.
PAN C. H., HEPLER L., ERLANDER A. P.
"Control of pH and carbohydrate addition in the Penicillin fermentation."
Dev. Ind. Microbiol.

PARK S. H., HONG K. T., LEE J. H., BAE J. C.
"On-line estimation of cell growth for glutamic acid fermentation system."

PASCUAL C., ALONSO A., GARCIA I, ROMAY C., and KOTYK A.
"Effect of ethanol on glucose transport, key glycolitic enzymes, and proton extrusion in Saccharomyces cerevisiae."
Biotechnology and Bioengineering.

PASTEUR L.
"Influence de oxygen sur le developpement de la levure et la fermentation alcoolique.
p 79.
Paris, June 28, (1861).

PEPPLER H. J.
"Yeast Technology."
Microbial Technology.
Eds. H. J. Peppler.

POLAKIS E. S., and BARTLEY W.
"Changes in the enzyme activities of Saccharomyces cerevisiae during aerobic growth on different carbon sources."
Biochem. J.
97, 284-297, (1965).

POSTMA E., VERDUYN C., SCHEFFERS A., and VAN DIJKEN J.P.
"Enzymatic analysis of the Crabtree effect in glucose-limited chemostat cultures."
Appl. Env. Micro.

RAINBOW C.
"Brewer's Yeast."
THE YEAST.
Academic press. Eds. Rose A. H., Harrison J. S.

RAJU G. K., COONEY C. L.
"Using newral networks for the interpretation of bioprocess data."
IFAC Modelling and Control of Biotechnical Process.
Colorado, USA.

RATLEDGE C.
"Yeast physiology - a micro-synopsis."
Bioprocess Engineering.
REDA K. D., THIEN M. P., GREASHAM R. L.  
"On-line robotics for multi-fermentor process monitoring and control."  
Advances in Laboratory Automation Robotica.  

REUSS M.  
"Measurement techniques for bioreactors."  
Biotechnology Focus 1 - Fundamentals, Applications, Information.  

RIEGER M., KAPPELI O, and FIECHTER A.  
"The role of limited respiration in incomplete oxidation of glucose by Saccharomyces cerevisiae."  
Journal of general microbiology.  

ROBBINS Jr. J. R. and TAYLOR K. B.  
"Optimisation of Escherichia coli growth by controlled addition of glucose."  

ROSE A. H., and HARRISON J. S.  
"Yeast and the environment."  

ROSE A. H., and HARRISON J. S.  
"The Yeasts. Metabolism and physiology of yeast."  

ROYCE P. N.  
"Structure monitoring of gas exchange in fermenter for control."  
(1993).

ROYCE P. N.  
"A discussion of recent developments in fermentatin monitoring and control from a practical perspective."  
Critical Reviews in Biotechnology.  

RUENGLERTPANYAKUL W., KONSTANTINOV K. B., YOSHIDA T.  
"Application of neural networks to variable estimation and stage identification in phenylalanine production."  
IFAC Modelling and Control of Biotechnical Process.  
Colorado, USA.  
SALMON P. M., BUCKLAND B. C.
"Monitoring and control of microbial fermentations."
IFAC Modelling and Control of Biotechnical Processes.

SCHAAFF I., HEINISCH J., and ZIMMERMANN F. K.
"Over-production of glycolytic enzymes in yeast."

SCHUGERL K.
"Common instruments for process analysis and control."
Biotechnology.
Volume 4, Measuring Modelling and Control.

SOLS A, GANCEDO C., DE LA FUENTE G.
"The Yeasts."
Edt. Rose A. H., Harrison J. S.

SONNLEITNER B., and KAPPELLI O.
"Growth of *Saccharomyces cerevisiae* is controlled by its limited respiratory capacity: Formulation and verification of a hypothesis."

SONNLEITNER B.
"Dynamics of yeast metabolism and regulation."

SONNLEITNER B, LOCHER G., FIECHTER A.
"Biomass determination. Minireview."

SOUMALAINEN H., NYMINEN T., OURA E.
"Aspect of cytology and metabolism of yeast."

STEPHANOPOULOS G., SAN K.
"Studies on on-line bioreactor identification."

STEUBE K., SPOHN U.
"Determination of intracellular dehydrogenase activities using flow injection analysis."
*Journal of biotechnology.* 33, 221-231, (1994)

STONE K., ROCHE F. W., and THORNHILL N. F.
"Dry weight measurement of microbial biomass and measurement variability analysis."
STRUDSHOLM K., EMBORG C., and SIGSGAARD P.
"Automatic containment sampling of recombinent Escherichia coli fermentations."

STRYER L.
"Biochemistry."

THORNHILL N. F., and ROYCE P. N. C.
"Modelling fermentations for control."

TURNER C.
"The application of a novel sampling device to the on-line analysis of fermentation broth."

TURNER C., THORNHILL N. F., and FISH N. M.
"A novel method for on-line analysis of the fermentation broth using a sampling device, microcentrifuge and HPLC."

VALERO F., LAFUENTE F. J., SOAL C., BENITO A., VIDAL M., CAIRO J., and VILLAVERDE A.
"Simultaneous on-line monitoring of intracellular b-galactosidase activity and biomass using flow injection analysis in Escherichia coli batch fermentations."

VAN UDEN N.
"Ethanol toxicity and ethanol tolerance in yeast."

VERDUYN C., POSTMA E., SCHEFFERS A., and VAN DIJKEN J.P.
"Energetics of Saccharomyces cerevisiae in anaerobic glucose-limited chemostat culture."

VON MEYENBURG K.
"Energetics of the budding cycle of Saccharomyces cerevisiae during glucose limited aerobic growth."
WANG H. Y., COONEY C. L., and WANG D. I. C.
"Computer-aided Baker's yeast fermentations."

WILSON J. A.
"Modelling fermentations for control."
Measurement and control for Bioprocesses.
Edt. Carr-Brion K. G.

WINKLER M.
"Time profiling and environmental design in computer-controlled fermentation and enzyme production."
Genetically engineered proteins and enzymes from yeasts: production control.
Ellis Horwood series in biochemistry and biotechnology.
Edt. Wiseman A.
96-146, (1991)

WU W-T, TSAO J-H, FAN C-P.
"On-line estimation of cell mass in glutamic acid production."
Journal of Fermentation and Bioengineering.

ZABRISKIE D. W.
Comprehensive Biotechnology.
'The principles of biotechnology: Science fundamental.'
Edtd. Merray Moo-Young. Pergamon press Ltd.

ZENG A-P, DECKWER W-D.
"Pathway analysis of oxygen utilisation and tricarboxylic acid cycle ativity in Saccharomyces cerevisiae growing on glucose."
Journal of Biotechnology.

ZHONG J-J., KONSTANTINOV K. B., and YOSHIDA T.
"Computer-aided on-line monitoring of physiological variables in suspended cell cultures of Perilla frutescens in a bioreactor."
Journal of fermentation and bioengineering.

ZIMMERMANN F. K.
"Glycolytic enzymes as regulatory factors."
Journal of Biotechnology.