UNIVERSITÉ DE MONTRÉAL

SIMULTANEOUS CONTROL OF GLUCOSE AND AMMONIUM CONCENTRATIONS DURING FED-BATCH CULTURE OF *Alcaligenes eutrophus* DSM 545

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SIMULTANEOUS CONTROL OF GLUCOSE AND AMMONIUM CONCENTRATIONS DURING FED-BATCH CULTURE OF *Alcaligenes eutrophus* DSM 545

présenté par: MODIRROUSTA Mostafa

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a été dûment accepté par le jury d'examen constitué de:

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DEDICATION

It is always easy to dedicate a piece of work to someone. It is even easier to make dedication to several persons and to be proud (of nothing of course). What all this means is: "I remember you" what is that: self-satisfaction. What is hard to do is to love them in the real life when there is still time. Nevertheless this work humbly is dedicated

To: Mitra, Mandana, Mom, and Dad

For a while in childhood, we attended a master;
for a while we were happy with our own mastery;
at the end of the story see what happened to us:
like water we entered and like wind we departed.

Khayyam Neishabouri

(Persian Poet)

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RÉSUMÉ

L'objectif principal de cette étude était d'étudier la possibilité de contrôler simultanément deux substrats, le glucose et l'ammonium, durant la phase de croissance d'*Alcaligenes eutrophus* DSM 545. Le produit intracellulaire était le biopolymère PHB (Poly-ß-hydroxybutyrate). Un schéma réactionel proposé antérieurement a constitué la base du développement mathématique du système de contrôle. Un mode d'opération en cuvée alimentée a semblé, d'autre part, approprié à cette fermentation.

Les concentrations de substrat ne pouvaient pas être mesurées en ligne. Les concentrations d'oxygène et du CO_2 dans les gaz à l'entrée et à la sortie, à l'aide d'un spectromètre de masse à quatre pôles, ont été traitées comme des variables mesurées. Les variables non-mesurées étaient suivies à l'aide d'un observateur asymptotique dérivé du modèle dynamique du système. Celui-ci a été transformé pour éliminer le terme cinétique inconnu. L'observateur a été appliqué à ce modèle transformé. Il a servi à estimer les concentrations de biomasse, de glucose, et d'ammonium à partir du taux de production de gaz carbonique.

La dynamique du système était hautement non linéaire et non stationnaire. Le taux de croissance spécifique a été considéré comme un paramètre dépendant du temps. La

théorie du contrôle non-linéaire a été utilisée pour tenir compte de toute la dynamique du système, et pas seulement d'une forme linéarisée de celle-ci. La méthode proposée, un contrôle linéarisant entrée-sortie a résulté en un système linéaire en boucle fermé sur les concentrations en glucose et en ammonium. Un estimateur était requis pour déterminer le taux de croissance spécifique à partir du taux de production de CO_2, information nécessaire aux calculs de la loi de contrôle.

Les données de quatre fermentations révèlent une production anormalement élevée de PHB pendant la phase de croissance sans limitation apparente de substrat. Le schéma réactionnel de départ a donc été corrigé pour tenir compte d'une production de PHB selon un ratio constant par rapport à la biomasse totale. Les algorithmes d'estimation et de contrôle conçus antérieurement demeuraient valables.

Le coefficient de rendement du CO_2 est apparu constant pour chaque fermentation prise séparément, mais différent d'une fermentation à l'autre. Cette observation s'expliquerait par la différence du ratio PHB/biomasse pour chaque fermentation. Les divers coefficients de rendement en CO_2 ont été utilisés pour estimer la biomasse et la concordance est bonne entre les concentrations mesurées et prédites.

Les résultats de la dernière fermentation ont démontré une bonne performance du système de contrôle adaptatif dans l'estimation de l'état (biomasse, glucose, ammonium)

et du taux spécifique de croissance lorsque les coefficients de rendement étaient corrects et constants. Un des paramètres clés pour une bonne estimation a été le débit d'air. Dans cette étude, il a été mesuré à l'aide d'un appareil à déplacement positif et a été contrôlé manuellement. Il est toutefois conseillé d'utiliser un appareil plus précis comme un débitmètre massique associé à une vanne de contrôle. Les raisons de la production inattendue de PHB durant la phase de croissance devraient également être identifiées. Il est recommendé d'appliquer cette méthode de contrôle à un schéma réactionnel découplé entre la croissance et la production si cette méthode de contrôle est à utiliser. D'autre part, un dispositif en ligne indépendant supplémentaire tel qu'un analyseur de biomasse ou un analyseur de glucose serait nécessaire. Des mesures en ligne des concentrations de biomasse et de PHB donneraient un contrôle plus fiable et d'application générale, puisque le modèle dynamique résultant ne dépendrait pas des coefficients de rendement et pourrait aussi être utilisé lorsque croissance et production ne sont pas découplées.

ABSTRACT

The principal objective of this study was to investigate the possibility of simultaneous control of glucose and ammonium during the growth phase of *Alcaligenes eutrophus* DSM 545. The intracellular product was the biopolymer PHB (Poly-β-hydroxybutyrate). A reaction network previously proposed formed the base of the mathematical development of the control system. Fed-batch culture was used.

The substrate concentrations could not be measured on-line. Therefore the inlet and outlet CO_2 concentrations were analysed using a quadrupole mass spectrometer allowing the CO_2 transfer rate to be exploited as the measured variable. The non-measurable variables were estimated by an asymptotic observer which was derived from the dynamical model of the system. The dynamical model of the system was transformed to eliminate the kinetic term which was assumed to be unknown. This observer was applied to this transformed model. This observer was used to estimate biomass, glucose, and ammonium concentrations from carbon dioxide production rate.

The dynamics of the system were highly nonlinear and nonstationary, and contained a time-varying parameter, the specific growth rate. Therefore nonlinear control theory was employed to benefit from the entire dynamics of the system, not just a linearized form of it. The proposed method was an input-output linearizing control resulting in a linear feedback system in glucose and ammonium concentrations. Another estimator was used to estimate the specific growth rate from CO_2 production rate data which was necessary for the control law calculation. Hence the control system became adaptive due to the adaptation of the specific growth rate.

When the data from four fermentations were analyzed, it was found that PHB production was unusually high during the growth phase with no apparent nutrient limitation. Thus the reaction network for the growth phase was revised such that PHB was produced as a constant ratio of the total biomass. However, previously conceived algorithms for estimation and control were still valid for the modified reaction network provided PHB-biomass ratio was constant.

The CO_2 yield coefficient was found to be constant for each individual fermentation, but differed from one fermentation to another. This was explained by considering the difference of the PHB-biomass ratio of each fermentation. These different CO_2 yield coefficients were used to estimate biomass which resulted in good concordance.

The results of the last fermentation demonstrated good performance of the adaptive control system, state (biomass, glucose, ammonium) estimation and the specific growth rate estimation when yield coefficients were correct and constant. One of the key parameters for a good estimation was the air flow rate. In this study it was measured using a positive displacement device and was manually controlled. It is recommended to use a more precise device such as a mass flowmeter coupled to a control valve, to measure and to control more precisely. The reasons behind the unexpected production of PHB during the growth phase should also be determined. It was recommended to apply this control method to a growth/production decoupled reaction network if the same control method is to be used. Otherwise one more independent on-line measurement, such as a biomass detector or glucose analyzer would be required. On-line measurements of the biomass and PHB concentrations would result in a more reliable and generally applicable control, because the resulting dynamical model would not depend on yield coefficients and could also be used when growth/product are not decoupled.

CONDENSÉ EN FRANÇAIS

Les polyhydroxyalkanoates (PHAs) tels que le poly-β-hydroxybutyrate (PHB) sont des inclusions intracellulaires que l'on trouve dans un grand nombre d'espèces bactériennes. Certains de ces composants peuvent être utilisés comme plastiques biodégradables. Ils ont en effet de bonnes propriétés mécaniques et sont complètement biodégradables. Malheureusement, le coût des PHAs est trop élevé pour qu'ils soient compétitifs avec leurs concurrents pétrochimiques. Un des moyens d'en réduire le coût de production est l'optimisation du procédé.

Alcaligenes eutrophus est la bactérie choisie pour la production de PHAs à courte chaîne. Cette opération est réalisée en cuve alimentée. Elle comprend une phase de croissance suivie d'une phase de production. Il a été établi que la cinétique de croissance est limitée par les concentrations de deux substrats (glucose et ammonium), à condition que les autres nutriments soient en excès. Le glucose et NH_4^+ sont les deux substrats de croissance les plus importants mais ils inhibent la croissance si leurs concentrations sont trop élevées. Si la concentration de ces substrats peut être maintenue dans la plage optimale, la croissance se fera à la vitesse maximum, ce qui réduira les dépenses de capital et d'opération.

Le principal objectif de ce travail était d'étudier la possibilité d'un contrôle simultané du glucose et de l'ammonium durant la phase de croissance de *A. eutrophus* DSM 545. Le produit de fermentation était le PHB. La stratégie du contrôle était bâtie sur un modèle dynamique utilisant un schéma réactionnel modifié qui avait été développé antérieurement.

Cependant, le glucose et l'ammonium ne pouvaient pas être mesurés en ligne. Leur concentration a donc été estimée à partir d'analyses fréquentes des gaz de sortie, et ce, à l'aide d'un spectromètre de masse à quatre pôles.

Le modèle dynamique a été transformé pour éliminer le terme cinétique inconnu. Un observateur asymptotique a été appliqué à ce modèle modifié pour estimer en ligne les concentrations de biomasse, de glucose et d'ammonium à partir du taux de transfert de CO_2.

Le succès de cet observateur repose sur l'hypothèse que les coefficients de rendement de CO_2 et des substrats étaient connus et constants. Cette hypothèse a été vérifiée par les données expérimentales de ce travail. Les coefficients de rendement de CO_2, de glucose et d'ammonium étaient constants pendant toute la durée de chaque fermentation prise individuellement. De plus, l'estimation de la concentration en biomasse à partir du taux de transfert de dioxyde de carbone concordait avec les mesures de masse sèche de biomasse. Le taux spécifique de croissance (μ) était considéré comme un paramètre dépendant du temps et inconnu. Sa valeur était requise par l'algorithme de contrôle proposé. C'est pourquoi elle a été évaluée à partir des données de taux de transfert du CO_2. Pour cela, trois méthodes ont été utilisées. Elles sont basées sur les équations de bilan du CO_2. L'une d'elle est une méthode récursive des moindres carrés; la seconde utilise un observateur et propose deux façons de régler son paramètre de conception. La troisième solution est une simple intégration de l'équation de bilan mais contrairement aux précédentes, requiert le coefficient de rendement du CO_2. Ces méthodes ont été appliquées aux données expérimentales. Aucune différence significative n'a été relevée entre la méthode récursive des moindres carrés et l'estimateur basé sur l'observateur, alors que la troisième méthode était sujette à des erreurs importantes, principalement pendant les vingt premières heures de fermentation. La méthode récursive des moindres

carrés a donc été choisie parce qu'elle minimise l'erreur dans l'estimation.

Après avoir analysé les résultats des trois premières fermentations, il s'est avéré qu'en dépit des hypothèses de départ (à savoir un faible rendement de PHB durant la réaction de croissance), le PHB comptait pour plus de 30% de la masse sèche de biomasse. Cependant le ratio PHB/biomasse était relativement constant durant chacune des fermentations. Cette information a été incorporée dans le schéma réactionnel adopté et, en conséquence, dans la réaction globale qui a résulté, les réactions de croissance et de production de PHB se produisaient simultanément à des vitesses proportionnelles. Ce nouveau schéma réactionnel a la même structure que le premier, à la différence près que les coefficients de rendement ont été calculés par rapport à la biomasse totale (biomasse active + PHB).

Cette loi de contrôle a été obtenue en linéarisant le modèle en boucle fermée, à l'aide d'une commande linéarisante par rétro-action. Son principe est d'imposer un comportement dynamique linéaire entre les variables contrôlées et les points de consignes.

Au cours de la dernière fermentation, l'observateur asymptotique, l'estimation de μ et les algorithmes de contrôle ont été utilisés. La fermentation a été démarrée en mode cuvée pour laquelle les coefficients de rendement nécessaires ont été calculés puis introduits dans l'algorithme de contrôle. Après l'étape en cuvée, l'ammonium était relativement bien contrôlé autour de 2g/L de consigne lorsqu'un problème technique relatif au débit d'air s'est produit. Quant au glucose, sa concentration a chuté à 8.8 g/L. Cette anomalie a été attribuée au faible coefficient de rendement utilisé par le contrôleur. C'est pourquoi un coefficient plus élevé fut choisi. À partir de ce moment, le glucose a été bien contrôlé jusqu'à ce que survienne l'incident technique. Le débit

d'air incorrect a été interprété comme une activité bactérienne accrue ou une consommation plus élevée de substrat. Plus de substrat que ce qui était consommé a donc été ajouté. C'est pourquoi les concentrations ont augmenté à cet instant.

Un programme informatique a été élaboré. Il acquiert les données d'un spectromètre de masse, effectue les calculs nécessaires à l'estimation des variables d'états et de μ et à la loi de commande, enfin il envoie les signaux de commande aux pompes de substrats. Une dernière fermentation en cuvée alimentée a prouvé la performance de l'observateur et de la commande linéarisante. Puisque le rapport PHB/biomasse changeait d'une fermentation à l'autre, cette dernière fermentation a été commencée en mode cuvée. En analysant les échantillons du début et de la fin de cette période, les coefficient de rendement de CO_2, de glucose, et d'ammonium ont été calculés. Ces valeurs ont été introduites dans le programme de contrôle. Une autre analyse, après environ 20 heures, a montré que l'ammonium était bien contrôlé entre 1.97 et 2.09 g/L pour un point de consigne de 2 g/L. Cependant la concentration du glucose a baissé à 8.8 g/L pour un point de consigne de 15 g/L. Il s'est avéré que le coefficient de rendement du glucose utilisé par le programme était inférieur à sa valeur effective. Un rendement plus grand a donc été entré dans le programme. Malgré certains problèmes techniques, les deux substrats ont été bien contrôlés pendant les 20 heures suivantes. La concentration du glucose était régulée entre 14.26 et 15.9 g/L, tandis que celle de l'ammonium était compris entre 1.87 et 2.10 g/L. Aprés environ 70 heures, le débit d'air nécssaire au calcul du taux de transfert de CO_2 n'était plus fiable à cause d'une défectuosité de l'appareil de mesure. À cause de ce problème, le reste de l'expérience a échoué.

Conclusions:

1) L'hypothèse qu'une petite production de PHB est associée à la croissance n'est pas toujours valable. Dans toutes les cultures en cuve alimentée, une quantité considérable de PHB a été produite durant la phase de croissance.

2) La quantité de CO_2 produite par masse de biomasse produite ($k_{co2/x}$) est restée à peu près constante durant la phase de croissance, même lorsque du PHB était produit.

3) La biomasse peut être estimée approximativement à partir du taux de production de dioxide de carbone.

4) Le taux spécifique de croissance peut être estimé à partir du taux de production de dioxide de carbone sans que le coefficient de rendement du CO_2 ne soit requis.

5) Les concentrations de substrats peuvent être maintenues simultanément dans une plage acceptable au voisinage des points de consigne à conditions que certaines conditions soient remplies. Si les coefficients de rendement sont constants, un bon contrôle simultané du glucose et de l'ammonium est possible.

6) Si le PHB produit n'est pas proportionnel à la biomasse totale, la mesure de la production de CO_2 seule ne sera pas suffisante parce que les coefficients de rendement apparents ne sont plus constants et que l'adoption d'un schéma de réaction global n'est pas réaliste.

Recommandations:

1) Les facteurs contrôlant la croissance et la production associée de PHB devraient être identifiés. Par exemple, différentes souches de *A. eutrophus* pourraient être testées en

mode cuvée alimentée. La concentration initiale de substrats pourrait être abaissée comme dans un réacteur chemostat pour déterminer si une concentration élevée en substrat peut affecter la production de PHB.

2) Le débit d'air doit être mesuré et contrôlé plus précisément puisque la base de l'observateur est le taux de production de dioxide de carbone, calculé à partir des mesures d'un spectromètre de masse, ainsi que le débit d'air dans le réacteur.

3) Pour une fermentation où la croissance et la formation du produit ne sont pas découplés au moins deux mesures indépendantes sont nécessaires à l'estimation, du moment que les coefficients de rendement sont connus. À cet effet, il est recommandé d'utiliser la biomasse et le taux de transfer de CO_2, ou le taux de transfer de CO_2 et le glucose.

4) Si les concentrations de PHB et de biomasse pouvaient être des variables mesurées, il n'y aurait pas nécessité de connaître les valeurs des coefficients de rendement puisque la matrice (K) (eq.3.4) disparaîtrait si le modèle était modifié. Ainsi, en mesurant les concentrations de biomasse et de PHB, l'algorithme d'estimation deviendrait plus robuste et ne serait pas affecté par l'incertitude ou la variation des coefficients de rendement dans le couple production de PHB - croissance.

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LIST OF SYMBOLS

C_1, C_2	:	Tuning parameters in μ estimator
C	:	Oxygen concentration in the liquid phase in the liquid phase of the fermentor (g/L)
$c_{l,i}$	:	Concentration of the component i in the liquid phase (g/L)
c^*	:	Equilibrium concentration (g/L)
D	:	Dilution rate (h^{-1})
D_1	:	Dilution rate due to glucose feed (h^{-1})
D_2	:	Dilution rate due to ammonium feed (h^{-1})
e_i	:	Error in control of the substrate i
F	:	Input vector in the mass balance formulation
F	:	Flow rate (g/h)
F_{CO2}	:	CO_2 flow rate ($g(CO_2)/h$)
f_1	:	Glucose flow rate into the fermentor (L/h)
f_2	:	Ammonium flow rate into the fermentor (L/h)
G	:	Air flow rate (L/min)
H	:	Henry coefficient (mol m^{-3} Pa^{-1})
k_{ij}	:	yield coefficient of the component i in the reaction j (g/g)
k_1,k_2	:	Reaction rate constants
k_{ac}	:	H_2CO_3 dissociation constant
k_la	:	Volumetric mass transfer coefficient (s^{-1})
K	:	Yield coefficient matrix and/or equilibrium constant
k_{CO2}	:	global yield coefficient of CO_2 per g of total biomass (g/g)
k_{S1}	:	global yield coefficient of S1 per g of total biomass (g/g)
k_{S2}	:	global yield coefficient of S2 per g of total biomass (g/g)

$k_{CO2/Xtot}$: yield coefficient of CO_2 per g of total biomass (g/g)

$k_{O2/Xtot}$: yield coefficient of O_2 per g of total biomass (g/g)

$k_{S1/Xtot}$: yield coefficient of S1 per g of total biomass (g/g)

$k_{S2/Xtot}$: yield coefficient of S2 per g of total biomass (g/g)

$k_{CO2/Xa}$: yield coefficient of CO_2 per g of active biomass (g/g)

$k_{S1/Xa}$: yield coefficient of S1 per g of active biomass (g/g)

$k_{S2/Xa}$: yield coefficient of P1 per g of active biomass (g/g)

$k_{CO2/P2}$: yield coefficient of CO_2 per g of PHB produced (g/g)

$k_{S1/P2}$: yield coefficient of S1 per g of PHB produced (g/g)

M_1 : Weight of the glucose reservoir (g)

M_2 : Weight of the ammonium reservoir (g)

M_{base} : Weight of the base flask (g)

M_{CO2} : Molecular weight of CO_2

N_λ : Approximate number of previous data considred in the Recursive least squares method

P_{tot} : Over head pressure (Pa)

P_t : Recursive least squares parameter

P1 : CO_2 concentration in the liquid phase of the fermentor (g/L)

P2 : PHB concentration in the liquid phase of the fermentor (g/L)

p : Fixed pole in μ estimation

Q : Output vector in the mass balance formulation and/or volumetric CO_2 production rate ($gCO_2/h/L$)

Q_{CO2} : Volumetric CO_2 production rate ($gCO_2/h/L$)

Q_m : Measured volumetric CO_2 production rate ($gCO_2/h/L$)

$\Re$: Real number set

r_i : Reaction rate (mol/L/s)

S1 : Glucose concentration in the liquid phase of the fermentor (g/L)

S2	:	Ammonium (NH_4^+) concentration in the liquid phase of the fermentor (g/L)
T	:	Sampling period (h)
t	:	Time (h)
V_{evap}	:	Volume loss due to evaporation (L)
$V_{sampled}$	:	Sampled volume (L)
V_{S1}	:	Volume fed from glucose reservoir (L)
V_{S2}	:	Volume fed from ammonium reservoir (L)
V_{base}	:	Volume fed from base flask (L)
V_{tp}	:	Molar volume of CO_2 at the operating pressure and temperature
x	:	Mole fraction
X_a	:	Active biomass concentration (g/L)
X_{max}	:	Maximum attainable biomass concentration in a given fed-batch culture (g/L)
X_{tot}	:	Total biomass concentration (g/L)
Z	:	Auxiliary variable (g/L)

Greek alphabet:

α	:	The variable used in μ estimation algorithm
γ	:	PHB biomass ratio (g/g)
Δ	:	Difference operator
δ	:	Perturbation
λ_i	:	Eigenvalues of the error dynamics
λ	:	Forgetting factor
μ	:	The specific growth rate (h^{-1})
ξ	:	State vector

ξ_i	:	State variable i (g/L)
Φ	:	The matrix of the reaction rates
ϕ_i	:	The rate of reaction i (g/L/h)
ω	:	Noise on data

Superscripts

$\bullet$	:	Time derivative
$\hat{}$	:	Estimated value
'	:	Derivative
*	:	Set point
$\bar{}$	:	Reduced variables (Eq 3.45)

Subscripts

in	:	into the fermentor
out	:	out of the fermentor
i,j	:	indices

LIST OF ABBRIVIATIONS

CDW	:	Cell dry weight (g/L)
CER	:	Carbon dioxide evolution rate (g/h)
CPR	:	Carbon dioxide production rate (g/h)
CTR	:	Carbon dioxide transfer rate (g/h)
DO	:	Dissolved oxygen concentration
EKF	:	Extended Kalman filter
KF	:	Kalman filter
MSM	:	Mineral salt medium
OD	:	Optical density of the culture broth
OTR	:	Oxygen transfer rate (g/h)
OUR	:	Oxygen uptake rate (g/h)
PHA	:	Polyhydroxyalkanoate acid
PHB	:	Poly-β-hydroxybutyrate acid
P(HB-co-HV)	:	poly-β-hydroxybutyrate and poly-β-hydroxyvalerate
RPS	:	Relative parametric sensitivity
TES	:	Trace element solution

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Chapter 1: Introduction

In North America, plastics account for about 7% by weight of municipal wastes. Although they can be recycled or incinerated, recycling and incineration may not be adequate. Petrochemical plastics are rarely biodegradable and are produced from finite resources. Therefore scientists have been searching for replacements. The ideal plastic in terms of "sustainable development" should possess the appropriate thermomechanical properties, and be recyclable, completely biodegradable and produced from renewable resources [Ramsay 1990].

Some biopolymers are known to meet these criteria [Anderson 1990]. In the mid 1970s Imperial Chemical Industries produced a biopolymer named Biopol (poly-β-hydroxybutyrate and hydroxyvalerate) which was a copolymer of PHA (polyhydroxyalkanoate) family. It is 100% biodegradable, potentially recyclable, and produced from renewable resources. It can be substituted for certain synthetic plastics.

1.1 What are PHAs?

PHAs are water-insoluble polyesters of alkanoic acids containing at least one hydroxyl group in addition to the carboxyl group [Steinbüchel 1991]. Their general formula is shown in Fig. 1.1. The polymer with $n=1$ and $R = CH_3$, poly-β-hybroxybutyrate or PHB, is the most common member of PHA family. It has been known since 1926 [Lemoigne 1926].

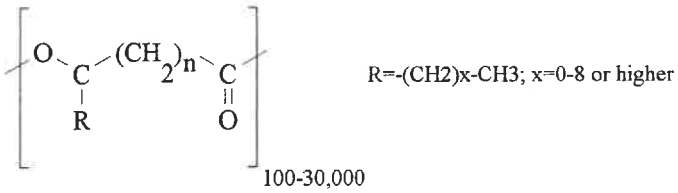

Figure 1.1 General structural formula of polyhydroxyalkanoic acids

PHAs are produced by a large variety of microorganisms especially bacteria [Brandl 1990]. They can also be produced synthetically [Steinbüchel 1991] but synthetic production processes are not yet commercially viable and may never be.

PHAs are a reserve of carbon and energy for bacteria [Byrom 1993]. Rapid PHA production may require limitation by an essential element (other than carbon) or its production may be independent of nutrient limitation, *Pseudomonas oleovorans*, [Ramsay *et. al.* 1991] or growth-associated, *Rhodospirillium rubrum*, [Brandl 1990].

1.2 Industrial production of PHA

Although the discovery of PHAs has opened new frontiers for the plastics industry, economic considerations have greatly restricted their usage. The economics of bacterial PHA production are governed by several factors, including product yield. So the selection of a particular microorganism or nutrient will have a crucial influence on cost [Byrom 93]. The complexity of the implemented technology and hence the capital cost of the plant and the ease or difficulty of product separation are also of extreme importance.

A vast range of research is aimed towards cost reduction in the production of PHAs. Selection of a cheap carbon source [Bertrand 1992], cloning and expression of genes involved in PHA biosynthesis into plants [Poirier 1992] or other organisms (e.g. *E. coli*) [Kim 1992], reactor design and its operation [Braunegg 1994], are examples of efforts to achieve this goal.

Zeneca[1], is the sole commercial producer of PHA and has chosen *Alcaligenes eutrophus* as its PHA production organism. A glucose-salt medium two-stage fed-batch fermentation is employed. Phosphate is used as the limiting substrate to induce the PHA production in the second phase after biomass has reached a certain concentration [Byrom 1990].

As has been discussed in detail in Johnson [1992] and Van Impe [1992] an optimal fed-batch process is achieved by the control of the reaction rate. If the fermentation is performed in two stages, depending on the reaction kinetics, different control strategies must be employed [Van Impe 1992].

1.3 Control Problems

In spite of a growing interest in fermentation processes in the last decades [Johnson 1987], the automization of industrial fermentors is still developing slowly. The main reasons, which also apply to PHA production process, are modelling, and measurement problems [Dochain 1990].

The dynamics of these processes are usually poorly known. Participating reactants and

---

[1] The biological products company resulting from the reorganization of the former ICI

products, and important reactions occurring during the process are rarely entirely known. Therefore these processes are always simplified during model development. Their dynamic behavior is often highly nonlinear and nonstationary.

In most industrial applications, only a subset of the biochemical and biological variables are available on-line from the sensors which must not be too expensive, but need to be reliable, reusable, and sterilizable. Frequently the controlled variables are not available on-line making control more difficult.

In addition to these two inherent problems, the reaction rate of most biotechnological processes depends on more than one reactant concentration. The growth phase of most PHA production processes has been established to follow double substrate dependent kinetics [Doi 1986, Ramsay 1990, Lee 1990]. In the process exploited by Zeneca, glucose and phosphate concentrations determine the reaction rate, provided that the other nutrients are in excess. Thus the optimization of these double-substrate reaction rate needs a multioutput control strategy.

1.4 Objective and methodology

The principal objective of this study was to investigate the possibility of simultaneous control of glucose and NH_4^+ concentrations during the growth phase of *Alcaligenes eutrophus* in fed-batch culture. This objective was selected such that upon its completion, optimization and kinetic studies of the process would be more easily achievable.

To achieve the principal objective, carbon dioxide transfer rate was proposed as the measured variable. Therefore two secondary objectives had to be accomplished. The

first secondary objective was to develop a state estimator based on the carbon dioxide transfer rate to give on-line estimates of the unmeasured variables (e.g. biomass, glucose, and ammonium concentrations). Since in this study no kinetic model was assumed, the second secondary objective was to estimate the specific growth rate, which was necessary for the proposed control algorithm, from the carbon dioxide transfer rate.

This study was a continuation of research concerning *Alcaligenes eutrophus*. This bacteria is of great interest and has been subject of numerous studies. Its response to certain carbon sources as well as the control of NH_4^+ concentration during the growth phase were studied at École Polytechnique de Montréal [Cornet 1993, Lefebvre 1992]. In the present work fed-batch culture of *Alcaligenes eutrophus* was investigated. Only homopolymer (PHB) production was studied using glucose as the carbon and energy source. The control of both glucose and NH_4^+ concentrations was achieved using a linearizing adaptive control strategy previously applied to the same process for single variable control (NH_4^+ concentration control) using the optical density of the culture broth as the measurable variable [Cornet 1993].

Off-gas analysis of the reactor outlet using a mass spectrometer was used to calculate carbon dioxide transfer rate which was the measured variable, because it was more frequently available and reflected well the biomass growth. While previously only one substrate concentration was controlled [Cornet 1993, Kim 1994], in this work an attempt was made to construct a control algorithm capable of controlling two substrate concentrations. Since glucose and NH_4^+ concentrations were not available on-line, an asymptotic observer was developed to estimate these concentrations from off-gas analysis data. Another estimator was also developed for the specific growth rate estimation, the time-varying parameter of the system, from off-gas analysis data. These two estimation algorithms were then used in conjunction with the linearizing control.

1.5 Contents

Chapter 2 is dedicated to a literature review on PHB production by *Alcaligenes eutrophus*, the reaction network of the process, fed-batch culture, control problems of fed-batch culture, and control methods including a section on state variable and parameter estimation. In Chapter 3, the dynamical model of the process is developed. State variable and parameter estimators are then derived from this model followed by a discussion of adaptive control law. Chapter 4 presents the materials and methods used in this study. The results of four fermentations conducted during this study are presented and discussed in Chapter 5. Conclusions and recommendations for future work are given in Chapter 6.

Chapter 2: Literature review

This chapter reviews the literature related to important topics of this study. The *Alcaligenes eutrophus* PHB production process by is covered and the important reactions of the system are summarized in the form of a reaction network. A description of fed-batch culture and its control problems in biotechnological processes follows. The control issues of nonlinear systems commonly found in biotechnology are presented next. Several methods of estimating system variables and parameters which are necessary when a full state measurement is not available are surveyed at the end of the chapter.

2.1 PHB production by *Alcaligenes eutrophus*

Alcaligenes eutrophus is a Gram-negative strictly aerobic rod. It was chosen by Zeneca for their commercial PHAs (P(HB-*co*-HV)) production, because of its ease of growth and ability to accumulate large amount of high molecular weight polymer [Byrom 1987].

Studies on different aspects of PHB production from glucose by *Alcaligenes eutrophus* have revealed that PHB production by *Alcaligenes eutrophus* is not highly growth associated, and that rapid PHB synthesis occurs when growth is restricted by the availability of a nutrient such as nitrogen, phosphorus, or oxygen [Lee, *et. al.* 1993, Mulchandani, *et. al.* 1989, Sonnleitner, *et. al.* 1979]. There are two pathways leading to PHB synthesis depending on the carbon source used, Fig. 2.1 [Doi 1988]. One of the differences is their response to nitrogen limitation. When glucose is the substrate, PHB synthesis is almost completely inhibited if $(NH_4)_2SO_4$ concentration exceeds 3 g/L (i.e. 0.82 g(NH_4^+)/L). In contrast, production of PHB from butyric acid is only slightly

inhibited by the presence of a nitrogen source [Doi 1988].

The nature of PHB accumulation by *Alcaligenes eutrophus* suggests that biomass production and PHB formation can be carried out separately. Different culture modes have been applied to the production of PHA including batch [Lee, *et. al.* 1991], chemostat [Lefebvre 1992, Lee, *et. al.* 1992, Bertrand 1992], and fed-batch [Byrom 1993, Cornet 1993, Kim, *et. al.* 1994]. Commercial PHB production by *Alcaligenes eutrophus* is conducted in two consecutive stages. Biomass is first cultivated without stimulating rapid PHB synthesis. When a key nutrient such as nitrogen is exhausted, PHB production is triggered. Since during the fermentation some nutrients are added without removing the products, this operation mode is called a fed-batch process.

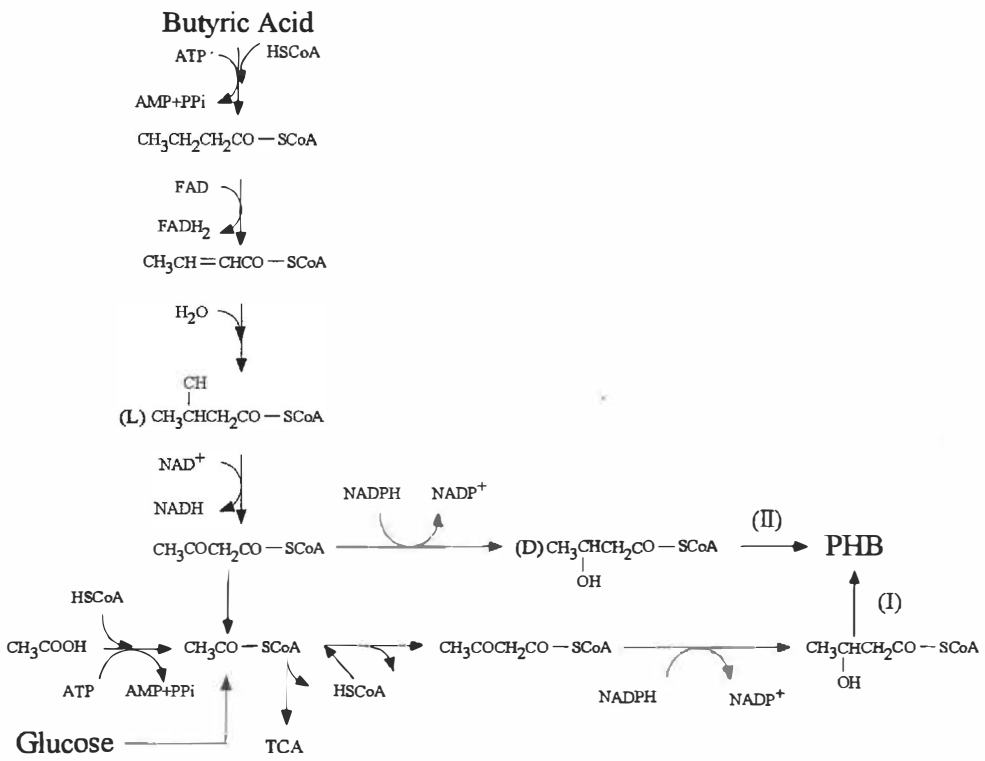

Figure 2.1 Pathways for the production of PHB [Doi 1988]

2.2 Reaction network of PHB production by *A. eutrophus*

The starting point of most control algorithms is a mathematical model of the system. In chemical engineering this model is usually obtained by applying mass and energy balances. If it is a reactive system, the contributing reactions must be known to develop the balance equations.

Hundreds of reactions participate in the growth of microorganisms. These reactions components are never all known. For practicality, the concept of a macroscopic reaction network was introduced. A reaction network comprises those reactions and components which have significant effects on the process kinetics.

The reaction network of the PHB production by *Alcaligenes eutrophus* results from the following information. Two limiting substrates are needed for the microbial growth (i.e. fructose or glucose as carbon source and ammonium as nitrogen source) [Repaski, *et. al.* 1976]. The intracellular production of PHB associated to the growth is negligible [Heinzle, *et. al.* 1980]. Both microbial growth and product formation yield gaseous carbon dioxide [Yamane 1993, Lefebvre 1992]. The respiratory quotient during the growth phase is constant and close to one [Bastin *et. al.* 1990].

Bastin and Dochain [1990] applied this knowledge to the PHB production process by *Alcaligenes eutrophus* from fructose. The process was described by the following reaction network:

$$S1 + S2 + C \xrightarrow{\phi 1} X_a + P1 + P2 \qquad (2.1)$$

$$S1 + C \xrightarrow[X_a]{\phi 2} P1 + P2 \qquad (2.2)$$

where X_a, *S1*, *S2*, *C*, *P1*, and *P2* stand for active biomass, fructose (carbon and energy source), ammonium, oxygen, CO_2, and PHB respectively. Biomass plays two different roles in these reactions. The first reaction is autocatalytic because the rate of this reaction is proportional to the cell concentration. In the second reaction biomass is necessary for the reaction but is not produced.

The first reaction involves the growth of biomass from two substrates assuming the other nutrients are in excess. Growth-associated production of PHB is not neglected. The second reaction describes PHB formation stimulated by ammonia depletion. These two reactions could happen simultaneously or consecutively, depending upon the mode of operation.

In a batch fermentation, since all nutrients necessary for the growth are in the reactor at the beginning, product formation follows growth if the ammonium concentration is depleted well before the carbon source. Otherwise, if there is no limitation, growth would dominate the entire fermentation. In continuous fermentation, depending on the substrate concentrations in the feed, PHB may or may not be produced. In the fed-batch process, a growth stage precedes the product formation. This two-step process is possible because PHB is mostly non-growth-associated. Thus with the hypothesis that PHB production is negligible during the growth phase, the reaction network presented by Equations (2.1) and (2.2) simplifies to:

$$S1 + S2 + C \xrightarrow{\phi 1} X_a + P1 \qquad (2.3)$$

$$S1 + C \xrightarrow[X_a]{\phi 2} P1 + P2 \qquad (2.4)$$

2.3.1 Fed-batch culture

Most industrial fermentation processes are operated in either batch or fed-batch mode. Over the past few years, fed-batch culture has been introduced in an increasing number of fermentations [Weigand 1981]. This is because theoretical analysis of many fermentations has revealed that extended or exponential fed-batch cultures are optimal [Agrawal 1989]. Fed-batch popularity has also increased due to improved control possibilities using computer-coupled fermentation systems [Hennigan *et. al.* 1982, Rolf *et. al.* 1982]. In this operation mode the reactor (normally a STR) is partially filled with reactants, and additional reactants are added successively, by a specific strategy, until the desired end products are formed [Yamane *et. al.* 1984]. Some of the most important reasons leading to the application of such systems are described by Yamane and Shimizu [Yamane *et. al.* 1984]. Those related to PHB production process are as follows.

Substrate (e.g. glucose) inhibition of the growth of biomass can be prevented by adding these substrates gradually at a rate comparable to which they are consumed. Moreover high cell concentrations can be achieved. In batch culture the whole quantity of substrate to yield high cell concentration (e.g. in excess of 100 g/L) must be added at the beginning. At such concentrations, some nutrients (e.g. glucose) would be inhibitory.

2.3.2 Classification of fed-batch techniques

Fed-batch culture has been classified in two main categories, with feedback and without feedback control [Yamane and Shimizu 1984] (Table 2.1).

Table 2.1 Classification of fed-batch operations in microbial processes

1. Without feedback control	**2. With feedback control**
Intermittent addition	Indirect feedback control
Constant rate	Direct feedback control
Exponentially increased rate	Constant-value control
Optimized	Optimal control
Others	

In fed-batch operation with direct feedback control, the concentration of the substrate in the reactor directly controls the addition rate of further substrate, whereas in the fed-batch operation with indirect feedback control, other system parameters such as dissolved oxygen (DO), pH, or respiratory quotient (RQ) are used. In either case the controlled variable is either kept at a constant level or changed based on some calculated trajectory. The other cases are self-explanatory.

2.3.3 Dynamical model of fed-batch cultivation

For the sake of simplicity, some realistic assumptions will considered in developing the dynamical model of a fed-batch process. The summary of these assumptions are listed henceforth.

1. The reactor is perfectly mixed (lumped system).
2. The biofilm effect is negligible.
3. The maintenance needs are negligible.

4. The specific growth rate is unknown.

The dynamical model is obtained by applying the mass balance principle for system variables (e.g. biomass, glucose, ammonium, CO_2) with the above assumptions. The system consists of M reactions and N components. The differential equation [Bastin *et. al.* 1990] associated with the ith component is:

$$\dot{\xi}_i = \sum_{j \sim i} (\pm) k_{ij} \, \phi_j - D\xi_i + F_i - Q_i \tag{2.5}$$

where:

ξ_i is the ith component concentration,

k_{ij} is the yield coefficient of the component i in the reaction j,

the positive sign is used for products and negative sign for reactants,

ϕ_j is the reaction rate of the jth reaction,

D is the dilution rate,

F_i is the mass flow rate in the reactor of the component ξ_i per reactor volume,

Q_i is the volumetric rate of mass outflow of the component ξ_i from the reactor in gaseous form per reactor volume.

The notation $j \sim i$ means the summation is taken on the reactions with index j which involve the component with index i,

2.3.4 The control of fed-batch cultures

The characteristics of the model (Eq 2.5) significantly affect the choice and performance of the control algorithm. This model is nonlinear due to dilution term ($D\xi_i$). Moreover, efforts at modelling the reaction rate as a function of system variables have resulted in various models each containing parameters to be identified. However practical difficulties in parameter identification of even a simple model such as Monod's [Nihtilä *et. al.* 1977, Holmberg 1982] put the usefulness of these models in doubt.

Measurement of state variables is still a challenging obstacle. There is either no sensor for them or the available methods and sensors are costly, non-sterilisable, and time consuming. For feedback control the value of the controlled variable must be known on-line. Therefore the lack of sensor provokes serious difficulties in biotechnological processes.

A notable difference between fed-batch and continuous mode is the inherent nonstationary nature of fed-batch systems. In fed-batch culture, the cell concentration, liquid volume and certain state variables change significantly during the course of the operation. Thus if the goal of a fed-batch culture is to exponentially accumulate a reaction product, some other state variable must follow an exponentially growing trajectory, which is characteristic of unstable behavior [Bastin *et. al.* 1990]. A study on the use of conventional PIDs controller has shown that due to this property the error of a PID controller also increases exponentially [Pomerleau 1990].

The aforementioned facts demonstrate that the dynamical model of a fed-batch culture is characterised by nonlinearity, nonstationary behavior, uncertainty in some parameters, and the presence of time-varying parameters such as μ. Because of these features, it is

an obvious field for application for adaptive linearizing control law [Bastin *et. al.* 1990]. More details about this type of control follow in the next section.

2.4 Control Methods

In a review of adaptive control strategies, Seborg *et. al.* [1986] have reported that adaptive control schemes provide systematic, flexible approaches for dealing with nonlinearities, uncertainties, and time-varying process parameters. Thus this type of control offers significant potential benefits for processes which may be poorly understood and/or vary in unpredictable ways. Biotechnological processes are good examples of this. Hence application of adaptive controllers are typically expected in this field.

Never-the-less why shouldn't other control strategies be applicable? According to Pomerleau [1990], control strategies mostly fall into four major classes, although not necessarily clearly separated. These are:

I- Classical control
II- Inferential and adaptive control
III- Optimal control
IV- Optimal adaptive control

Substrate concentration control using conventional PID controllers has been shown to be unstable for fed-batch cultures [Pomerleau 1990].

The second category, adaptive controllers, generally could be described as a strategy to estimate model parameters on-line (from some on-line measurements) and then adjust

the controller settings based on current parameter estimates. Seborg *et. al.* [1986] have surveyed three popular design strategies: self-tuning controllers, stability-based methods (e.g., model reference adaptive control), and pole placement techniques. Each one has its own benefits and disadvantages. The second strategy has been exploited for fed-batch culture [Dochain *et. al.* 1985, Montgomery *et. al.* 1985, Shioya *et. al.* 1985].

In the third type of controller an objective function (e.g. productivity) is optimised, then the control law which meets this objective is obtained. For fed-batch culture this class of control is usually not possible often because a suitable model of the system is not available. Even when a model is available, the problem of singular arc limits the usage of optimal control for fed-batch cultures. A singular arc is a period of optimal control when the optimal trajectory of manipulated variable vanishes from the equation giving the control law. This happens frequently in fed-batch fermentations [San *et. al.* 1984c]. For simple kinetic models (such as the Monod type) there is an answer to this problem, but for more complicated cases either there is no solution, or it is derived using more complicated techniques (e.g. Green's theorem) [Ohno 1976].

The fourth class consists of those controllers which rely on an estimation procedure (adaptation) and an optimal trajectory. These two are interactively used because system parameters are either uncertain or time-varying. The updated parameters determines the optimal trajectory for the next interval of time.

Optimal control theory appears to be *a priori* well suited for solving optimization problems of fed-batch cultures. From the early 70's, a number of theoretical works have concentrated on this issue [e.g. Fishman *et. al.* 1974]. However, the practical implementation of optimal control strategies is subject to great complexities. In addition precise knowledge of the process kinetics is required particularly the analytical structure

of the fermentation parameters (e.g. specific production rate or specific growth rate).

Aware of this common problem in biotechnology, Dochain and Bastin [Bastin 1990] constructed an adaptive control strategy which has certain interesting properties. Its adaptive aspect deals with the parameter uncertainty of the system (time-varying parameters such as the specific growth rate). The nonlinearity of the system dynamics was taken into consideration. Based on a general transformation law applied to their proposed general dynamical model for biotechnological processes, they have been able to eliminate the kinetic term in the model. Therefore the state estimation part of this control strategy has no need to any prior knowledge of the process kinetics. Moreover, its implementation is much more simpler than an optimal controller.

A flowchart showing different parts of this adaptive controller is presented in Figure 2.2. In this figure, estimation deals with both state estimation (reconstruction of those state variables which are not measured on-line), and parameter estimation (e.g. the specific growth rate). The estimation task could be performed with regard to two different philosophies. In the first approach estimation of state variables and model parameters are performed in the same manner. For example augmented Extended Kalman Filters can be developed and employed [Stephanopoulos 1982]. In the second approach one algorithm is used for parameter estimation and another independent one for the estimation of state variables [Dochain 1984].

When estimation is done during a data acquisition cycle, Figure 2.2, new values of the manipulated variables must be calculated. Some of the most commonly applied techniques in nonlinear control problems are: the minimum variance method, full state feedback linearization, and input-output linearization. Detailed discussions of these

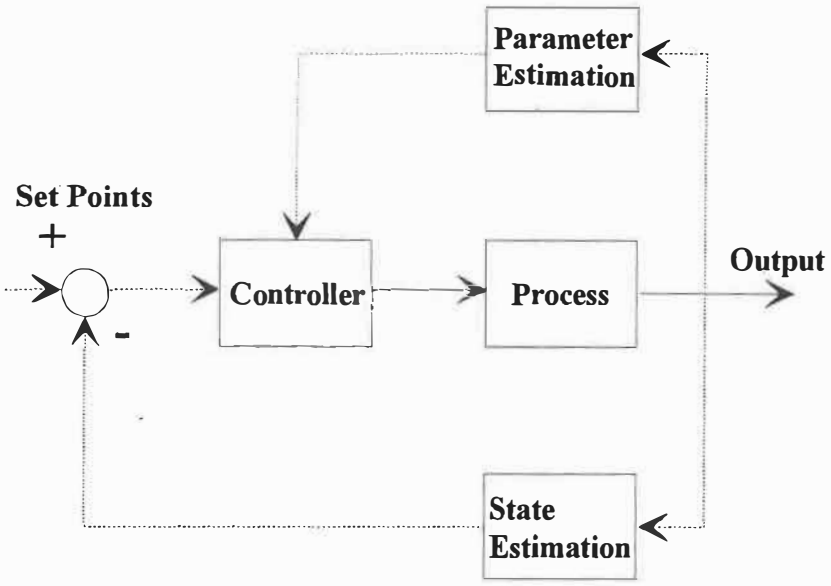

Figure 2.2 Adaptive Control Schema

methods is found in Isidori [1989]. Dochain [1992] compared full state and input-output linearization techniques and concluded that from practical point of view, the latter is more interesting due to their lower complexity and, in consequence, lower dependence with respect to the modelling of the process kinetics. In [Dochain 1984], an application of the minimum variance methods is given. Comparing his results with those obtained in [Chen *et. al.* 1991] for a similar dynamical model, it becomes clear that these two methods are not completely different. One (minimum variance method) could be considered as a special case of another (input-output linearization technique).

2.5 Estimation techniques

Any control or optimization based on the fermentation process key variables can not be implemented unless these variables can be measured or estimated on-line to provide the necessary information required by the controller or optimizer. In spite of the ongoing efforts to develop sensors that can be used to detect those key parameters, the availability and reliability of many of these instruments are very limited. Thus it is necessary to provide the necessary information by estimating those parameters from other parameters which are relatively simple and easy to measure. In this section some of the used and proposed algorithms for state variable and parameter estimation, are presented.

The available estimation methods can be classified as [Pons 1992]:

- I- Estimation through Macroscopic Balances
- II- Modern Estimation Techniques:
  - a- Deterministic Techniques
  - b- Stochastic Estimation Techniques

The first class comprises the traditional material and energy balance methods. Energy balances have played a less important role than the material balances since the fermentation processes take place in aqueous solutions and the heat of fermentation is relatively small in most cases.

There are two different approaches to material balances. The first approach is based on the concept of conservation of mass and the overall chemical reaction stoichiometry [Cooney *et. al.* 1977, Wang *et. al.* 1977]. In this method a certain composition of biomass is assumed and stoichiometric coefficients (yield coefficients) are also assumed. The elemental balance equations are normally written for C, N, O atoms. However the elemental composition of biomass is usually unknown. This method has been applied to PHB production by *Alcaligenes latus* [Heinzle *et. al.* 1991]. The estimated results differed significantly from those obtained by analysis.

The second approach to material balances proposes estimation of biomass concentration and growth rate through an on-line material balance [Zabriskie *et. al.* 1976, 1978]. An application of this method is the estimation of specific growth rate from carbon dioxide transfer rate (CTR) data. The mathematical derivation of the estimation equation is presented in Section 3.5.3. This method has two inherent drawbacks. It is assumed that noise-free data may be obtained but this is not often true. Moreover, it usually ends in a statement containing an integration term. Since measured variables are frequently noisy, integration is contaminated.

The modern estimation techniques have found many applications in biotechnology and research is continuing in this area. Observers are among the most well known methods in this class. Kalman filters (KF) for linear systems and their extended form (EKF) for non-linear systems are widely implemented. When this method, which is in a sense an

optimal observer, is applied to non-linear systems, it suffers from certain deficiencies, particularly the problem of tuning the vast set of parameters which sometimes have no physical correspondence from which reasonable values can be guessed. And particularly its stability is not proven in a general case.

Under this category one also can find the least squares and recursive least squares techniques. One application of the recursive least squares method is presented when μ is estimated from CO_2 transfer rate (Section 5.5).

Considering the drawbacks of EKF, Dochain [1986] introduced the notion of asymptotic observer for a rather wide class of biotechnological processes. This technique was demonstrated to be stable and convergent under a series of mild conditions [Dochain *et. al.* 1985]. It has been successfully applied to laboratory scale fermentors [Bastin 1990, Cornet 1993, Pomerleau 1990, Pomerleau 1992]. The EKF is complicated and needs a great amount of mathematical manipulation whenever new data is available. In addition, even for very simple cases it may give biased results [Dochain 1986]. In contrast the asymptotic observer technique is simple to implement, stable and convergent. In addition it does not need any kinetic model of the fermentation. For these reasons the asymptotic observer technique was chosen for state variable estimation in this work.

The estimation of the specific growth rate can be achieved by several methods including the recursive least squares method, an observer based method, and direct calculation with material balance equations. These methods are assessed in Section 5.5.

Chapter 3: Mathematical Development

To achieve the objectives of this study, the principles of adaptive controller needed to be tailored to the actual system in study. The controller was built on a mathematical model of the system. However, the state variables of the system are not readily measured on-line, except for the dissolved oxygen concentration. The estimation component reconstructs the nonmeasured variables from available on-line measurements. Finally, the specific growth rate (μ) is estimated in order to complete the knowledge of the system allowing control law calculation.

Mathematical manipulation of different parts of the control loop is presented in this chapter. The chapter is organized in the same sequence as the calculations. It starts with a material balance of the important components to obtain the dynamical model. A brief discussion on the selection of measurable variables is given, followed by the state variable estimation algorithm from CO_2 transfer rate data. Parameter estimation methods are then presented. Finally, the control law for maintaining the two substrate concentrations at desired set points is obtained by applying the principles of input-output linearizing feedback control.

3.1 Dynamical model

In this section the general dynamical model presented in Section 2.3.3, is rewritten in detail for the reaction network (2.1) and (2.2). The resulting differential equations are the basis of the estimation and control algorithms. They form the so called state space model in control theory.

To begin the reaction network (Eq 2.1 and 2.2) will be analyzed. This is more general than the reaction network represented by Eq 2.3 and 2.4 in that growth and production are not separated i.e. the small amount of PHB production expected in the presence of ammonium is not neglected. The mass balance principle is applied to glucose, ammonium, carbon dioxide, oxygen, and PHB as shown below:

Accumulation = Reaction (consumption or production) + (Input - Output)

The mass balance yields:

$$\begin{bmatrix} \dot{X}_a \\ \dot{S}_1 \\ \dot{S}_2 \\ \dot{P}_1 \\ \dot{P}_2 \\ \dot{C} \end{bmatrix} = K\,\Phi - D \begin{bmatrix} X_a \\ S_1 \\ S_2 \\ P_1 \\ P_2 \\ C \end{bmatrix} + \begin{bmatrix} 0 \\ D_1 S_{1in} \\ D_2 S_{2in} \\ 0 \\ 0 \\ Q_{in} \end{bmatrix} - \begin{bmatrix} 0 \\ 0 \\ 0 \\ Q_{CO_{2out}} \\ 0 \\ 0 \end{bmatrix} \tag{3.1}$$

where:

X_a is active biomass (total biomass - PHB) concentration,

S_1 is glucose concentration,

S_2 is NH_4^+ concentration,

P_1 is CO_2 concentration in the liquid phase,

P_2 is PHB concentration,

C is oxygen concentration in the liquid phase,

K is the matrix of yield coefficients,

Φ is the matrix of reaction rates,

D is the global dilution rate,

Q_{in} is the oxygen transfer rate to the reactor per reactor volume,

$Q_{CO2\,out}$ is the carbon dioxide transfer rate out of the reactor per reactor volume.

The separation of feeding reservoirs for glucose and ammonium also divides the dilution rate term in two. D_1 and D_2 are the dilution rates from glucose and ammonium feeding reservoirs.

$$D_1(t) = \frac{f_1(t)}{V(t)}$$
$$D_2(t) = \frac{f_2(t)}{V(t)} \qquad (3.2)$$
$$D(t) = D_1(t) + D_2(t)$$

where f_1 and f_2 are feeding rates of glucose and ammonium, respectively.

In a rather comprehensive study of this system [Bastin *et. al.* 1990] it was shown that this system is observable with <u>any two</u> on-line measurements. The demonstration is not given here, however the key element of the reasoning is the rank of the matrix K. For example oxygen and carbon dioxide could be used as two measurements, because the rank of the K matrix is <u>two.</u> However the respiration quotient[1] (RQ) of this bacterium during the growth phase is rather constant when using glucose as the carbon and energy source, Figure 3.1, which means these two measurements are not independent and consequently not sufficient for state reconstruction. The solution is either to use another set of measurements or to look for some additional information.

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[1]RQ is the ratio of the carbon dioxide evolution rate and the oxygen uptake rate

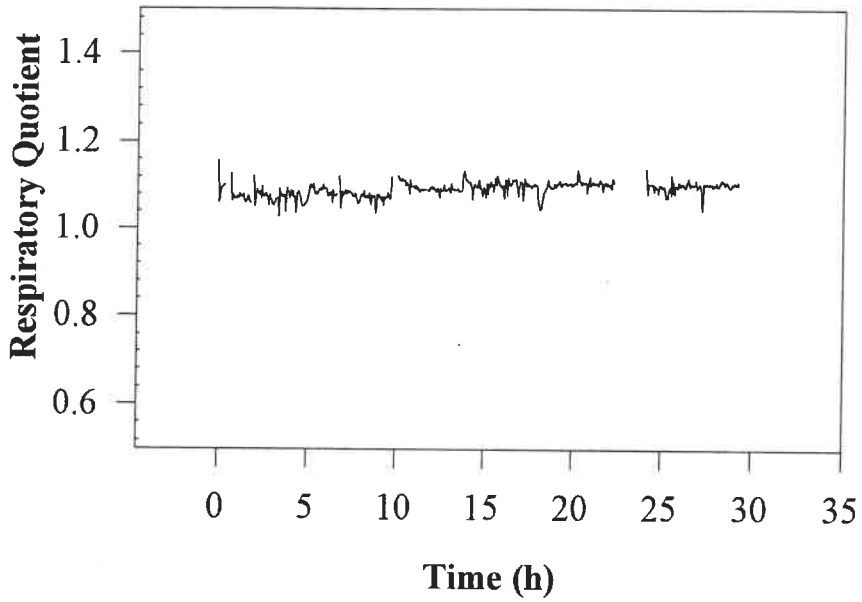

Figure 3.1 Respiratory Quotient calculated for Fermentation CII

As discussed previously the yield coefficient of growth associated PHB production is known to be low. Thus the assumption of a negligible growth associated PHB production is a sound assumption in a two-stage fed-batch culture. Therefore the reaction network (2.1) and (2.2) turns into a growth phase reaction (2.3) and a product formation phase reaction (2.4):

$$S1 + S2 + C \xrightarrow{\phi 1} X_a + P1 \qquad (2.3)$$

$$S1 + C \xrightarrow[X_a]{\phi 2} P1 + P2 \qquad (2.4)$$

The dynamical model of the growth phase for this modified reaction network is:

$$\begin{bmatrix} \dot{X}_a \\ \dot{S}_1 \\ \dot{S}_2 \\ \dot{P}_1 \\ \dot{C} \end{bmatrix} = \begin{bmatrix} 1 \\ -k_{S_1/X_a} \\ -k_{S_2/X_a} \\ k_{P_1/X_a} \\ -k_{C/X_a} \end{bmatrix} [\phi_1] - D \begin{bmatrix} X_a \\ S_1 \\ S_2 \\ P_1 \\ C \end{bmatrix} + \begin{bmatrix} 0 \\ D_1 S_1 in \\ D_2 S_2 in \\ 0 \\ Q_{O_2 in} \end{bmatrix} - \begin{bmatrix} 0 \\ 0 \\ 0 \\ Q_{CO_2 out} \\ 0 \end{bmatrix} \qquad (3.4)$$

For this model since the rank of the yield coefficient matrix is *one*, a *single* measurement suffices for reconstructing the entire system. In the next section the choice of an on-line measurable variable is discussed.

3.2 On-line measurable variables

The possible choices available in the course of this work were on-line measurement of pH, temperature, and dissolved oxygen concentration of the culture broth, the gaseous composition of inlet and outlet of the reactor, and the air flow rate into the reactor. Optical density of the culture broth was also available (manually) for each sample taken (usually every 2 to 3 hours).

To select the measurable variable of this study, the possibilities were examined for the information they could afford for the special task of double substrate concentration control. In many studies, pH is used to supply ammonia for the microorganism (e.g. Kim *et. al.* 1994). However NH_4^+ concentration of the culture broth was not controlled at a preset value.

In another study [San *et. al.* 1984b] pH was used as on-line data from which the volumetric rate of biomass production ($\frac{dX}{dt}\frac{1}{V}$) was estimated. However such information was not sufficient for the purpose of this work. Substrate concentrations still needed some other estimation method.

Optical density is another on-line measurement which is often used to monitor the biomass concentration. Recently it was used to control ammonium ion concentration in a PHB production process [Cornet 1993]. However biomass concentration is not always directly related to optical density in this process (Figure 3.2). Therefore basing the control strategy on optical density cannot be recommended, particularly during the PHB production stage where there is a poor relationship between biomass concentration and optical density.

Since exhaust gas data were available more frequently and there was a close stoichiometric relationship between CO_2 production rate (or O_2 production rate) and microbial activity [Yamane 1993], it was considered as a plausible possibility and is assessed in Section 5.4. A quadrupole mass spectrometer affords precise gas analysis [Oeggerli *et. al.* 1995] and one was available for this study.

Dissolved oxygen and oxygen composition of the gas phase of the reactor were used for the same system as that used in this study for a single variable control [Bastin *et. al.* 1990]. For two reasons dissolved oxygen was not used in this work. Firstly, noise and bias in CO_2 transfer rate data (*CTR*) is of the order that in the exit gas carbon dioxide analysis, while the relative amount of noise in the oxygen transfer rate (*OTR*) is one or two orders of magnitude greater than the noise in the raw oxygen analysis because the *OTR* is calculated as a small difference between two large quantities since ambient air contains a large amount of oxygen and relatively little CO_2 [Royce *et.al.* 1992,Tremblay

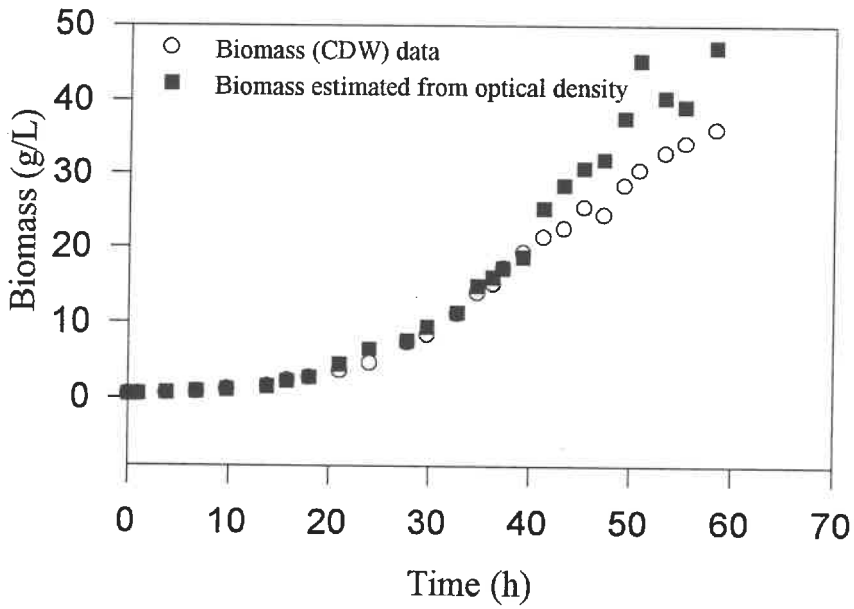

Figure 3.2 Insufficiency of optical density representation of biomass. Data from Fermentation CI.

1992]. Secondly, OTR and CTR data are not independent and do not provide more information because RQ is constant, Section 3.1. Therefore CTR was chosen instead of OTR.

3.3 Asymptotic observer

The substrate concentrations and the specific growth rate must be available to the control law. In this study substrate concentrations were not measured on-line and no model was assumed for the specific growth rate. Therefore they were estimated on-line. There is a tendency to separate the state variable estimation (e.g. glucose and ammonium concentrations) and system parameter estimation (e.g. μ). In this section state estimation is addressed. The asymptotic observer method was selected for this purpose because of its proven convergence, stability, and because it is specific growth rate independent (Section 2.5).

3.4 Formulation of the observer

The asymptotic observer was derived from a transformed form of the dynamical model, Eq 3.4. The transformation was applied to a more compact form of Eq 3.4, written using matrix notation:

$$[\dot{\xi}] = K\,\Phi_1 - D\xi + F - Q \tag{3.5}$$

where ξ is the vector of state variables, F and Q are the input and output mass transfer rate per reactor volume vectors , and K is the same as the yield vector in Eq 3.4.

The transformation was used to eliminate the kinetic term, Φ_1, in Equation 3.5. The transformation law used for elimination of the kinetic term is based on a linear combination of state variables. State variables are divided in two groups, ξ_a and ξ_b. Then an auxiliary variable, Z, is defined:

$$Z = A_0 \cdot \xi_a + \xi_b \tag{3.6}$$

where the matrix A_0 is the unique solution of Eq 3.7:

$$A_0 \cdot k_a + k_b = \mathbf{0} \tag{3.7}$$

The following partition was applied in this work to separate the measured and unmeasured variables, which leaded to a similar dynamics to that of Eq 3.5 with corresponding terms given below:

$$\begin{aligned}
&\xi_a = P_1 && \textit{and} && \xi_b^T = [X_a \;\; S_1 \;\; S_2]^T \\
&k_a^T = k_{CO_2/X_a} && \textit{and} && k_b^T = [1 \;\; -k_{S_1/X_a} \;\; -k_{S_2/X_a}]^T \\
&F_a^T = \mathbf{0} && \textit{and} && F_b^T = [0 \;\; D_1 \cdot S_{1,in} \;\; D_2 \cdot S_{2,in}]^T \\
&Q_a^T = CTR && \textit{and} && Q_b^T = [0 \;\; 0 \;\; 0]^T
\end{aligned} \tag{3.8}$$

The dynamics of the new auxiliary variable (i.e. Z) was calculated by eliminating ξ_a and ξ_b between Equations 3.6 and 3.5:

$$\dot{Z} = -D(t) \cdot Z(t) + A_0 \cdot (F_a - Q_a) + (F_b - Q_b) \tag{3.9}$$

Hence if the entities of Z vector are estimated and then Equation 3.6 is solved for ξ_b, state variable estimation is achieved without knowledge of the kinetic term.

This left the question of how to estimate Z. The yield coefficients of this process were assumed to be known, and the dynamical equation was the summary of important knowledge of the system, thus a state observer was proposed.

In contrast to the extended Luenberger and Kalman observers which are exponentially convergent, the asymptotic observer is a state observer which is asymptotically convergent [Bastin *et. al.* 1986]. This property enables this kind of observers to be used even if the model is not exponentially observable. This observer is applied to the transformed model to avoid the kinetic term. The resulted asymptotic observer for Eq 3.9 has virtually the same structure than that of Eq 3.9. The only difference is the presence of estimated variables instead of the real ones.

$$\begin{aligned} d\hat{Z}/dt &= -D(t) \cdot \hat{Z}(t) + A_0 \cdot (F_a - Q_a) + (F_b - Q_b) \\ \hat{\xi}_b &= \hat{Z} - A_0 \cdot \hat{\xi}_a \end{aligned} \tag{3.10}$$

The discretized version of Equation 3.10 employing a first order Euler approximation becomes:

$$\hat{Z}(t+1) = (1 - T \cdot D(t)) \cdot \hat{Z}(t)) - T \cdot CTR \cdot A_0 + T \cdot F_b \qquad (3.11)$$

where T is the sampling period.

The dilution rate is calculated from Eq 3.12:

$$D(t) = \frac{d(V)}{dt} \cdot \frac{1}{V} \qquad (3.12)$$

where $d(V)/dt$ is the feed expression obtained from volume data. The volume at each instant is calculated as:

$$V(t+1) = V(t) + \sum_{t}^{t+1} V_{S_1} + \sum_{t}^{t+1} V_{S_2} + \sum_{t}^{t+1} V_{base} - \sum_{t}^{t+1} V_{sampled} - \sum_{t}^{t+1} V_{evaporated} \qquad (3.13)$$

The derivative term in Eq 3.12 was approximated by a method different from Euler approximation. This was necessary since the calculation of a volume derivative at the instance t, having two points $V(t)$ and $V(t-1)$, is not accurate enough especially when the feeding rate is high. Therefore a derivative using three points was used (Eq 3.14, Lagrange Interpolating Polynomials method) [Burden *et. al.* 1993].

$$f'(x_i) \approx f(x_{i-1}) * \frac{(x_i - x_{i+1})}{(x_{i-1} - x_i)(x_{i-1} - x_{i+1})}$$
$$+ f(x_i) * \frac{2x_i - x_{i-1} - x_{i+1}}{(x_i - x_{i-1})(x_i - x_{i+1})} \qquad (3.14)$$
$$+ f(x_{i+1}) * \frac{(x_i - x_{i-1})}{(x_{i+1} - x_{i-1})(x_{i+1} - x_i)}$$

where $f'(x)$ is the time derivative of volume and x represents time.

3.5 Parameter estimation

The kinetic term (Φ_l) is the product of biomass concentration and specific growth rate (μX). The knowledge of the specific growth rate will be shown to be necessary for the control law calculation adopted in this work. In this study the estimation of μ is separated from estimation of the system state, by eliminating the unknown parameter (specific growth rate) from the space state model.

The estimation of μ was based on CO_2 transfer rate since it was the measured variable. Several algorithms have been proposed for the estimation of μ. Two are based on a gaseous product formation rate (e.g. CO_2) [Dochain 1986, Bastin *et. al.* 1986]. In the next section these algorithms, their tuning and another algorithm using mass balance principles, are discussed.

3.5.1 First Algorithm

Under the assumptions of quasi steady-state condition and negligible dilution effect, the volumetric CO_2 transfer rate can be expressed as:

$$Q = k_{CO_2/X} \cdot \mu \cdot X \tag{3.15}$$

where:

Q is the CO_2 transfer rate per unit of ungassed liquid volume in the reactor

$k_{CO2/X}$ is the yield coefficient.

The time derivative of this equation with some rearrangement is:

$$\begin{aligned} \dot{Q} &= k_{CO_2/X}\dot{\mu} X + k_{CO_2/X}\mu \dot{X} \\ &= k_{CO_2/X}\mu X \frac{\dot{\mu}}{\mu} + k_{CO_2/X}\mu X \frac{\dot{X}}{X} \\ &= Q\left(\frac{\dot{\mu}}{\mu} + \frac{\dot{X}}{X}\right) \end{aligned} \tag{3.16}$$

From the biomass balance we have:

$$\frac{\dot{X}}{X} = \mu - D \tag{3.17}$$

By introducing a new variable (α) and regrouping the terms that were in brackets, Eq 3.16, an analogous equation to the biomass balance results:

$$\alpha(t) = \mu(t) + \frac{\dot{\mu}(t)}{\mu(t)}$$
$$\dot{Q}(t) = \alpha(t) \cdot Q(t) - D(t) \cdot Q(t) \tag{3.18}$$

The proposed algorithm by Dochain [Dochain 1986] originates from Equation 3.18 which in its continuous form is:

$$\frac{d\hat{Q}(t)}{dt} = \hat{\alpha}(t) \cdot Q_m(t) - D(t) \cdot Q_m(t) + C_1 \cdot Q_m(t) \cdot [Q_m(t) - \hat{Q}(t)]$$
$$\frac{d\hat{\alpha}(t)}{dt} = C_2 \cdot Q_m(t) \cdot [Q_m(t) - \hat{Q}(t)] \tag{3.19}$$
$$\frac{d\hat{\mu}(t)}{dt} = -\hat{\mu}(t) \cdot [\hat{\mu}(t) - \hat{\alpha}(t)]$$

where:

C_1 and C_2 are design parameters and constant,

Q_m is the measured CO_2 transfer rate from off-gas data available on-line,

and the variables with (^) are estimated ones.

Such a structure was chosen for enhanced convergence and stability. The estimation is driven by the observation error ($Q_m(t)-\hat{Q}(t)$). The measurement estimate is updated via a differential equation (3.19) which includes the non-linear structure of equation (3.18) where α is replaced by its on-line estimate, and a term proportional to the

observation error.

By using a first order Euler approximation these equations can be written in discrete form as:

$$\begin{aligned}
\hat{Q}(t+1) &= \hat{Q}(t) + T \cdot Q_m(t) \cdot [\hat{\alpha}(t) - D(t) + C_1 \cdot (Q_m(t) - \hat{Q}(t))] \\
\hat{\alpha}(t+1) &= \hat{\alpha}(t) + C_2 \cdot T\, Q_m(t) \cdot (Q_m(t) - \hat{Q}(t)) \\
\hat{\mu}(t+1) &= \hat{\mu}(t) - \hat{\mu}(t) \cdot T \cdot [\hat{\mu}(t) - \hat{\alpha}(t)]
\end{aligned} \tag{3.20}$$

where T is the sampling period. The stability and convergence of this algorithm has been exhaustively studied elsewhere [Dochain 1986].

3.5.1.1 Tuning of Parameters C_1 and C_2

The continuous version of the above algorithm has been proven to have bounded estimation error provided that C_1 and C_2 are positive [Dochain 1986]. Moreover if:

$$C_2 < \frac{C_1^2}{4} \tag{3.21}$$

the algorithm converges without oscillation.

A modification of the tuning has been discussed in [Pomerleau and Perrier 1990]. The proposed strategy is an analogue pole assignment method to fix the convergence rate of

the algorithm but since the dynamical equation is not linear there is no constant pole. However by trying to keep a constant pseudo-pole throughout the course of time the convergence rate of the algorithm may also be kept constant [Pomerleau *et. al.* 1990]. To demonstrate this concept the error dynamics of this system is developed below.

$$\begin{bmatrix} \tilde{Q}_{(t+1)} \\ \tilde{\alpha}_{(t+1)} \end{bmatrix} = \begin{bmatrix} 1-TC_1Q_m(t) & TQ_m(t) \\ -TC_2Q_m(t) & 1 \end{bmatrix} \begin{bmatrix} \tilde{Q}_{(t)} \\ \tilde{\alpha}_{(t)} \end{bmatrix} + \begin{bmatrix} 0 \\ \alpha(t+1) - \alpha(t) \end{bmatrix} \tag{3.22}$$

where the variables with (~) are the errors (difference between estimated and real values):

$$\begin{aligned} \tilde{Q}(t) &= \hat{Q}(t) - Q_{real}(t) \\ \tilde{\alpha}(t) &= \hat{\alpha}(t) - \alpha_{real}(t) \end{aligned} \tag{3.23}$$

The pseudo-poles of the error dynamics are the eigenvalues of the coefficient matrix on the right hand side of Eq 3.22:

$$\lambda_i = \frac{(2-C_1TQ_m \pm \sqrt{\Delta})}{2} \qquad (i=1,2) \tag{3.24}$$

To insure non-oscillating convergence, λ_1 and λ_2 must be real, positive, and inside the unit circle. These conditions results in certain restrictions:

$$\begin{aligned} \lambda_i \in \Re \quad &\therefore \quad C_2 \leq \frac{C_1^2}{4} \\ 0 < \lambda_i < 1 \quad &\therefore \quad C_1 < \frac{2}{T\,Q_m} \end{aligned} \tag{3.25}$$

Throughout the fermentation the increase in Q_m results in the changes of the positions of λ_1 and λ_2 inside the unit circle if C_1 and C_2 are constants. Consequently, the convergence rate of the error dynamics varies due to the presence of Q_m in Eq 3.24. In order not to violate the convergence conditions, Eq 3.25, the tuning of the system must be done according to the highest expected value of Q_m. Since in a fed-batch culture the maximum amount of Q_m is reached at the end of the fermentation, the convergence at the beginning will be slow because of tuning with regard to the maximum value of Q_m [Pomerleau *et. al.* 1990]. Therefore it is proposed to use time varying design parameters which assure constant convergence rate for the whole fermentation. It is achievable by fixing a double pole, p, provided C_1 and C_2 are time-varying, Eq 3.26.

$$\begin{aligned} \Delta = 0 \;\Rightarrow\; C_2 &= \frac{C_1^2}{4} \\ C_1 &= \frac{2\,(1-p)}{T\,Q_m} \end{aligned} \tag{3.26}$$

where p is the double pole, provided $\Delta = 0$.

3.5.2 Second Algorithm

The second method for the estimation of μ is a least squares method [Dochain 1986]. This is applicable because the equation:

$$\dot{Q}(t) = \alpha(t)\, Q(t) - D(t)\, Q(t) \tag{3.27}$$

in its discretized form (Eq 3.28) is linear in regard to α:

$$Q(t+1) = Q(t) + \alpha(t)\, T\, Q(t) - D(t)\, T\, Q(t) + \omega(t) \tag{3.28}$$

where ω represents errors due to measurement noise. The recursive least squares algorithm becomes:

$$\hat{\alpha}(t+1) = \hat{\alpha}(t) + T\, P_t\, Q_m(t)\, (Q_m(t+1) - Q_m(t)\, [1 - T\, D(t) + T\, \hat{\alpha}(t)]) \tag{3.29}$$

where P_t updates are calculated from Eq 3.30:

$$P_t = \frac{P_{t-1}}{\lambda} \left[1 - \frac{(T\, Q_m(t))^2\, P_{t-1}}{\lambda + (T\, Q_m(t))^2\, P_{t-1}} \right] \tag{3.30}$$

The algorithm is initialized with $P_0 >> 0$ and $0 < \lambda <= 1$, usually between 0.95 and 1.00. Equations 3.29, 3.30, and the updates of μ and Q (Eq 3.20) form the estimator.

In Equation 3.30, λ is a forgetting factor to allow the tracking of the time-varying α. For a given forgetting factor λ, the parameter estimator yields an estimate based approximately on the last N_λ samples of data where:

$$N_\lambda \approx \frac{1}{1-\lambda} \tag{3.31}$$

Thus, a value of $\lambda=0.95$ yields a parameter estimate based essentially on the last $N_\lambda=20$ samples of data [Hang *et. al.* 1993].

3.5.3 Third Algorithm

One of the presented estimation methods in Section 2.5 takes advantage of the material balance principle to calculate the specific growth rate and not to estimate it. In this section the specific growth rate is calculated from the carbon dioxide transfer rate by this method.

The CO_2 mass balance equation in the dynamical model of the process, Eq 3.4, is:

$$\dot{P}_1 = k_{CO_2/X}\, \mu X - D\, P_1 - Q_{CO_2,out} \tag{3.32}$$

where $Q_{CO2,\ out}$ is the volumetric CO_2 transfer rate:

$$Q_{CO_2,out} = \frac{F_{CO_2}}{V} \tag{3.33}$$

where F_{CO2} is the transfer rate of CO_2 expressed in g(CO_2)/h. If in Equation 3.32 the derivative of P_1 and the dilution term DP_1 are neglected compared to the other terms, and Equation 3.33 is plugged into Equation 3.32, the relationship between F_{CO2} and μ results:

$$F_{CO_2} = k_{CO_2/X}\ \mu\ X\ V \tag{3.34}$$

Rearranging the biomass mass balance (Eq 3.4) gives:

$$\mu = \frac{1}{XV} \cdot \frac{d(XV)}{dt} \qquad or \qquad \mu XV = \frac{d(XV)}{dt} \tag{3.35}$$

By replacing ($\mu\ X\ V$) from this Equation into Equation 3.34:

$$F_{CO_2} = k_{CO_2/X} \cdot \frac{d(XV)}{dt} \tag{3.36}$$

and integrating the resulted Equation, $X\ V$ product may be expressed in terms of F_{CO2}:

$$(XV) = (XV)_0 + \frac{1}{k_{CO_2/X}} \int_{t_0}^{t_f} F_{CO_2} \cdot dt \tag{3.37}$$

When XV and $d(XV)/dt$ from Equations 3.37 and 3.36 are plugged into Equation 3.35, the final formula for calculating the specific growth rate from F_{CO2} data results:

$$\mu(t) = \frac{F_{CO_2}(t)}{k_{CO_2/X}(XV)_0 + \int_{t_0}^{t_f} F_{CO_2} \cdot dt} \tag{3.38}$$

which is an on-line formula to calculate the specific growth rate, μ, from off gas analysis data if the yield coefficient is known.

3.6 Adaptive input-output linearizing control

The control problem of this study has the following properties:

(I) the dilution rates are the control variables,

(II) the state variables glucose, ammonium, and biomass concentrations are estimated on-line,

(III) the specific growth rate is estimated on-line,

(IV) glucose and ammonium concentrations are the controlled variables.

Now that an on-line procedure for the determination of the state variables and the specific growth rate had been elaborated, it was necessary to develop the adaptive control law. The parameter estimation procedure was coupled with the control law to update the parameter of the dynamical model. In this way it was possible to compensate for the time-varying property of this parameter (μ).

In linearising control the closed loop system was designed to result in a globally linear function. The necessary condition for a full-state linearization has been discussed elsewhere [Isodori 1989]. The dynamical system of Equation 3.4 does not meet that condition. However the linearization is still achievable for the controlled state variables (glucose and ammonium). This feedback input-output linearization has some advantage. It is less complex than full-state linearization, and in consequence, less dependent on the modelling of process kinetics [Dochain 1992].

The selected control law in this study is an input-output linearizing method. In other words the dynamics of the controlled states are linearized by the feedback law. Figure 3.3 depicts the essential parts of the controller used in this study.

The control law is based on an input-output model of the controlled variable dynamics which can be obtained by rearranging Eq 3.4:

$$\begin{bmatrix} \dot{S}_1 \\ \dot{S}_2 \end{bmatrix} = \begin{bmatrix} -k_1 \\ -k_2 \end{bmatrix} (\mu \cdot X_a) - (D_1 + D_2) \cdot \begin{bmatrix} S_1 \\ S_2 \end{bmatrix} + \begin{bmatrix} D_1 & 0 \\ 0 & D_2 \end{bmatrix} \begin{bmatrix} S_{1,in} \\ S_{2,in} \end{bmatrix} \tag{3.39}$$

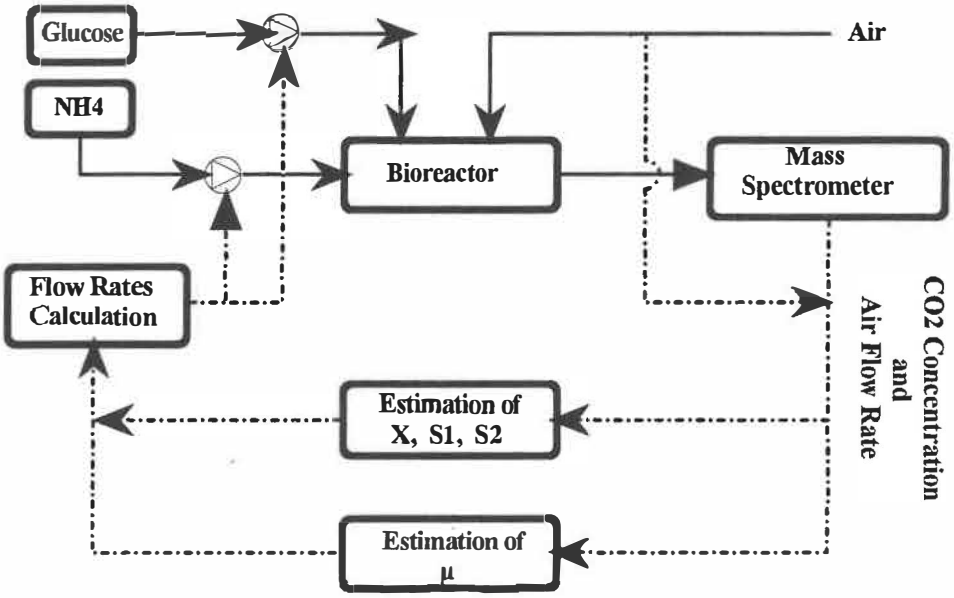

Figure 3.3 Adaptive controller flowchart

A first order reference model was chosen for both S_1 and S_2 errors which decay exponentially (λ_1 and λ_2 are positive).

$$\frac{d(S^*_1 - S_1)}{dt} + \lambda_1 (S^*_1 - S_1) = 0$$
$$\frac{d(S^*_2 - S_2)}{dt} + \lambda_2 (S^*_2 - S_2) = 0 \tag{3.40}$$

where λ_1 and λ_2 are constant tuning parameters, and S^*_1 and S^*_2 are the constant set points (i.e. $d(S^*_i) / dt = 0$).

The convergence of these continuous error dynamics is guaranteed as long as λ_1 and λ_2 are positive. The discretized form of the above equations is:

$$e_1(t+1) = (1 - \lambda_1 T) \cdot e_1(t)$$
$$e_2(t+1) = (1 - \lambda_2 T) \cdot e_2(t) \tag{3.41}$$

where e_1 and e_2 are the regulation errors ($e_i = S^*_i - S_i(t)$).

To have stable, non-oscillating regulation error dynamics, the poles of Equation (3.41) must be positive, and located on the real axis and inside the unit circle:

$$\begin{aligned} 0 < (1-\lambda_1 T) < 1 \\ 0 < (1-\lambda_2 T) < 1 \end{aligned} \tag{3.42}$$

or:

$$0 < \lambda_1 < \frac{1}{T} \qquad \textit{and} \qquad 0 < \lambda_2 < \frac{1}{T} \tag{3.43}$$

The derivatives of S_1 and S_2 in Equation 3.39 are replaced with their expressions from Equation 3.40 and after solving the resulting equations for D_1 and D_2 (manipulated variables), the control law becomes:

$$D_i = \frac{f_{i,in}}{V} = \lambda_i \, \Delta(\bar{S}_i) + \bar{k}_i \, \mu \, X_a + \bar{S}_i \left[\frac{\sum_{j=1}^{j=2} \lambda_j \, \Delta \bar{S}_j + \mu \, X_a \sum_{j=1}^{j=2} \bar{k}_j}{1 - \sum_{j=1}^{j=2} \bar{S}_j} \right] , \quad (i \in \{1,2\}) \tag{3.44}$$

where:

$$\bar{k}_i = \frac{k_1}{S_{2,in}}$$

$$\bar{S}_i = \frac{S_i}{S_{i,in}} \tag{3.45}$$

$$\Delta\bar{S}_i = \frac{(S^*_i - S_i)}{S_{i,in}}$$

3.7 Concluding Remarks

In this chapter different blocks of the designed closed loop control were mathematically developed and derived from the available knowledge about the process. Non-linear control and estimation theories were applied to fulfill a multioutput adaptive controller suitable for the growth phase of the PHB production process. In chapter 5 the performance of this controller will be evaluated using the results of a number of fermentations to substantiate and verify each part of the controller.

Chapter 4: Materials and Methods

4.1 Microorganism and inoculum

The microorganism used in this work was *Alcaligenes eutrophus* DSM 545. It was maintained on glucose agar plates at 4°C, and was subcultured monthly . The inoculum used in all fermentations was prepared in two steps in shake flasks. Inocula were prepared by cultivating one or two colonies of *A. eutrophus* at 30°C and 200 RPM in two 500 mL shake flasks each containing 50 mL of mineral salts medium[1] (Tables 4.1 and 4.2). This first step usually took 2 to 3 days. After that, the contents of the two 500 mL shake flasks were transferred into two 2 L shake flasks each containing 450 mL of MSM for a total inoculum of 1 L. The second step was rapid. The reactor was inoculated within 24 hours.

The medium composition of the inoculum was the same for all experiments except for the initial glucose and ammonium concentrations. In Fermentations Expo, CI, and CII, 10 g glucose and 2.73 g ammonium per litre were used while for Fermentation CIII, 12 g glucose and 2.88 g ammonium per litre were used. MSM was autoclaved in three separate different groups (Table 4.1) and cooled to room temperature before mixing to prevent undesired reactions between different ions.

---

[1]MSM

Table 4.1 Mineral Salt Medium (MSM) composition used for seed culture

	Substance	Concentration (g/L)
Group I	$MgSO_4$. $\bullet 7H_2O$	0.20
	TES[1]	1.0 mL/L
Group II	$CaCl_2 \bullet 7H_2O$	0.01
	NH_4-Fe(III)-citrate	0.06
	H_2SO_4	1 drop
Group III	$Na_2HPO_4 \bullet 7H_2O$	9.94
	KH_2PO_4	0.83

Table 4.2 Trace Element Solution (TES) composition[2]

Mineral Salt	Concnetration (g/L)
$ZnSO_4 \bullet 7H_2O$	0.10
$MnCl_2 \bullet 4H_2O$	0.03
H_3BO_3	0.30
$CoCl_2 \bullet 6H_2O$	0.20
$CuSO_4 \bullet 5H_2O$	0.01
$NiCl_2 \bullet 6H_2O$	0.02
$NaMoO_4 \bullet 2H_2O$	0.03

4.2 Fed-batch culture medium

Polypropylene glycol 2000 (PPG) was used as an antifoam agent (0.5 mL/L). Initially

[1]Trace Element Solution, Table 4.2

[2]Ramsay *et. al.* 1990c

the fermentor contained glucose and PPG which were sterilized in the reactor. MSM and ammonium sulphate were sterilized in four separate flasks, added to a single flask (6 L) after cooling, and added to the glucose solution in the reactor before the inoculation. The final MSM concentration in the reactor was same composition as in the inoculum except that there was only 3.76 g/L of $Na_2HPO_4 \bullet 7H_2O$ and 0.83 g/L of KH_2PO_4 since the pH was controlled (i.e. there was less need for buffering capacity). The initial conditions, volume and substrate concentrations, for the four fermentations are tabulated in Table 4.3.

Table 4.3 Initial conditions, substrate concentrations and volume

	Expo	CI	CII	CIII
Volume(0)	7.74	9.00	8.69	8.67
Glucose(g/L)	11.29	14.10	12.93	20.90
NH_4^+(g/L)	3.02	2.13	2.14	2.65

Glucose and ammonium sulphate were fed separately into the fermentor. The glucose reservoir contained concentrated glucose. The concentration used for each experiment is tabulated in Table 4.4. Total volume of this reservoir was about 3 L. The second reservoir contained ammonium sulphate in a volume of about 1.5 L (Table 4.4).

Table 4.4 Substrate feeding concentrations for different fermentations

Fermentation	Glucose (g/L)	NH_4^+ (g/L)
Expo	400.00	32.19
CI	380.95	63.00
CII	384.51	65.00
CIII	450.00	130.91

All fermentations began with a batch phase leading to the fed-batch phase. During the batch phase, initial substrates available in the reactor were consumed to specified level. For Fermentations Expo, CI, and CII these final concentrations were zero for glucose, and 2 g/L for NH_4^+. The approach of these conditions was detected by a rise in dissolved oxygen concentration at which point feeding started. The batch phase Fermentation CIII lasted until glucose and ammonium concentrations were close to their set points of 15 and 2 g/L respectively.

4.3 Fermentation equipment and data acquisition system

All fed-batch fermentations were performed in an 18 L Bioengineering fermentor (Bioengineering, Wald, Switzerland). Figure 4.1 illustrates the fermentor, measuring devices and data acquisition system used during this study. The fermentor was connected to a console (not shown in Fig. 4.1) comprising the necessary modules to monitor and/or to control pH, temperature, dissolved oxygen, agitation, and the air flow rate.

4.3.1 Environmental variables

The pH sterilizable probe (Ingold AG, Industrie Nord Urdrf/Zürich) was calibrated before the sterilization of the fermentor and was adjusted for possible drift after the sterilization but before inoculating. The pH was regulated at 7.0 $\pm$ 0.2 by addition of sulphuric acid (20% vol/vol) and sodium hydroxide (20% wt/vol).

The dissolved oxygen concentration (DO) was monitored by a sterilisable Ingold (Ingold AG, Industrie Nord Urdrf/Zürich) polarographic oxygen probe. During the fermentations, DO was maintained above 30% saturation except when specified.

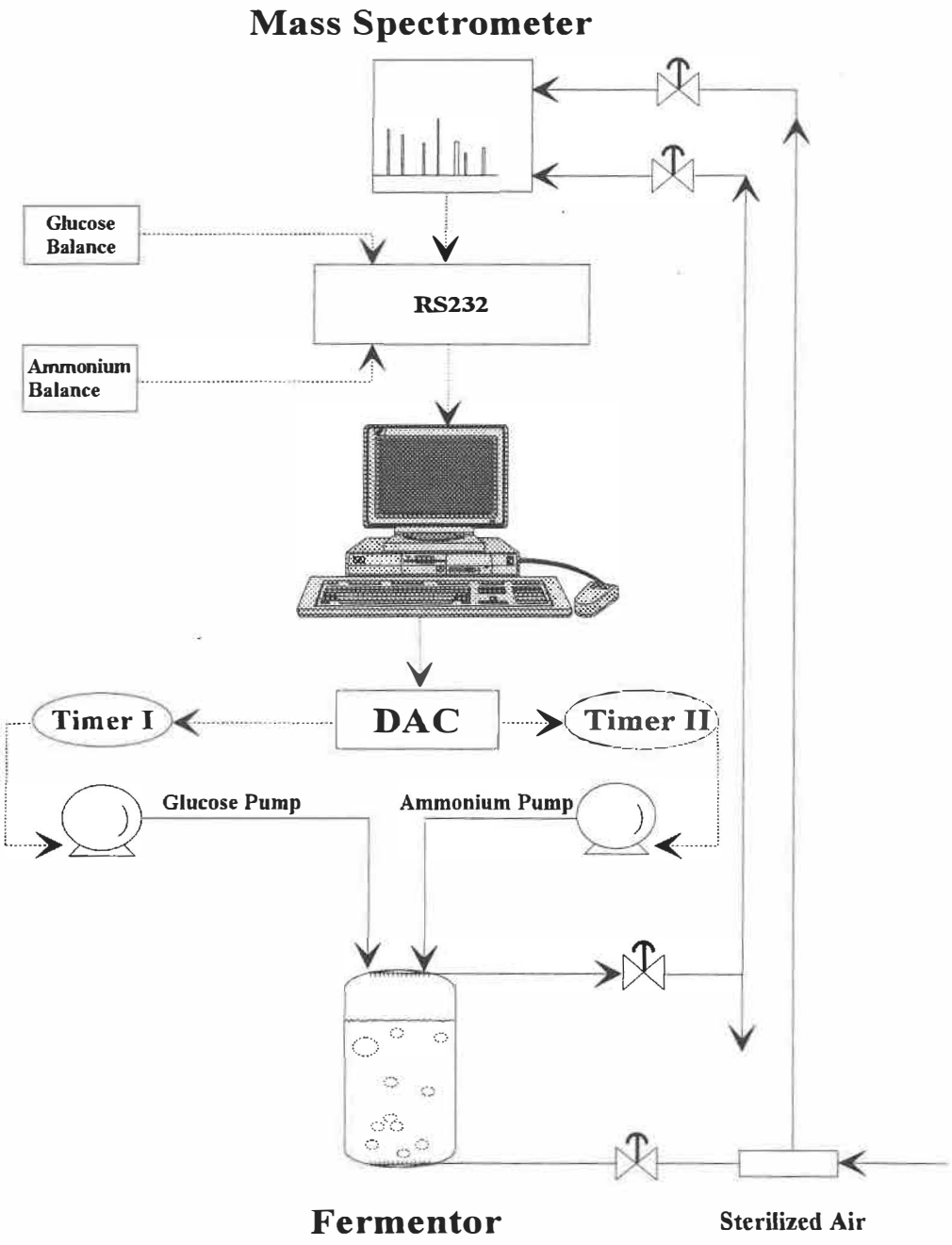

Figure 4.1 Control system configuration

The temperature of the culture broth was measured with a thermocouple and was automatically regulated at 30 $\pm$ 0.1 °C by adjusting the ratio of cold water and steam inside the fermentor jacket.

The air flow rate was monitored by a rotameter and measured more precisely by a positive displacement device. Excessive foam was detected visually and eliminated by addition of sterile PPG.

4.3.2 Employed variables in control and estimation algorithms

The variables used in the control and estimation algorithms were the carbon dioxide transfer rate, the substrate flow rates, the substrate reservoir weights, and the current culture volume.

The carbon dioxide transfer rate was calculated as in Eq a.15 (Appendix I). For this purpose the gaseous outflow of the fermentation was separated into two lines. One line provided a flow rate of 100-150 mL min^{-1} to a mass spectrometer (Micromass PC, VG Instrument Inc. Middlewich UK), type VG Quadrupole SX200F, while the other exhausted the surplus to the atmosphere. The average sampling period for mass spectrometer data was 6 min. The CO_2 and N_2 concentrations in the inlet and outlet of the fermentor were transferred via an RS232 cable to an IBM 486DX computer where all the calculations (estimation and control law) were made.

The CO_2 concentration in the liquid phase, necessary in the state estimator, was not measured. However, as discussed in Appendix I, this value was approximated supposing the CO_2 concentration in the outlet flow was proportional to the CO_2 liquid phase concentration (Henry's law). Possible discrepancies between estimated and real

CO_2 concentrations were neglected because this variable had little effect on the transformed state variables (Eq 3.6).

The control action for the glucose and ammonium input flow rates was applied to two variable speed Watson-Marlow peristaltic pumps, equipped with a 101U/R auto-control drive module. A two-zone calibration strategy was used to calibrate the peristaltic pumps used for substrate addition. The first zone corresponded to those flow rates which were less than pump's lowest limit. A timer was used for each pump in connection with the control program. Lower flow rates were obtained by adjusting period of each time cycle during which the pumps were actuated. The second zone corresponded to the higher flow rates and gave a continuous flow as it did not require a timer. These two zones were linear. The calibration was repeated before each fermentation. The numerical values of the substrate flow rates were transmitted to a data acquisition card (LabMaster DMA, Scientific Solutions, Inc., Solon, Ohio) which sent signals of 4-20 mA to the pumps.

The substrate flow rates used in the calculations were originally the same as that calculated by the control law. However technical and programming problems occurred during Fermentations Expo, CI, and CII. During Fermentation CIII these values were recalculated from the difference in the feeding reservoir weights to avoid these problems. These real data were then exploited by the control program. The reservoir weight were available via two RS232 port cables which connected the reservoir balances (Ohaus, model GT 8000) to the data acquisition system.

The volume of the fermentor at each instant was calculated by integrating the volume of the added substrates from the previous period until that instant. The volume change due to sampling or acid/base addition was also taken into account. These changes were

entered into the program manually. Evaporation was assumed to have little contribution to volume change except during the fermentor sterilization process where the evaporated volume was measured by condensing the outlet line of the fermentor in a graduated cylinder which already contained certain volume of cold water.

4.3.3 Control program

The program for data acquisition and calculation of the control law was written in Turbo Pascal version 6.0 (Borland International Inc). Inputs and outputs of the control program are tabulated in Table 4.5.

Table 4.5 Input and Output of the control program

Input	Output
Air Flow Rate (G)	Glucose feeding Rate for the next sampling period (f_1)
Off Gas Analysis, $(N_2 \& CO_2)_{in,\ out}$	
Glucose Reservoir Weight (M_1)	Ammonia Feeding Rate for the next sampling period (f_2)
Ammonia Reservoir Weight (M_2)	
Base Flask Weight (M_{base})	
Sampled Volume (V_{sam})	

The flowchart in Figure 4.2, shows the flow of calculations. Initial values of the tuning parameters (Initialization block) are tabulated in Table 4.6.

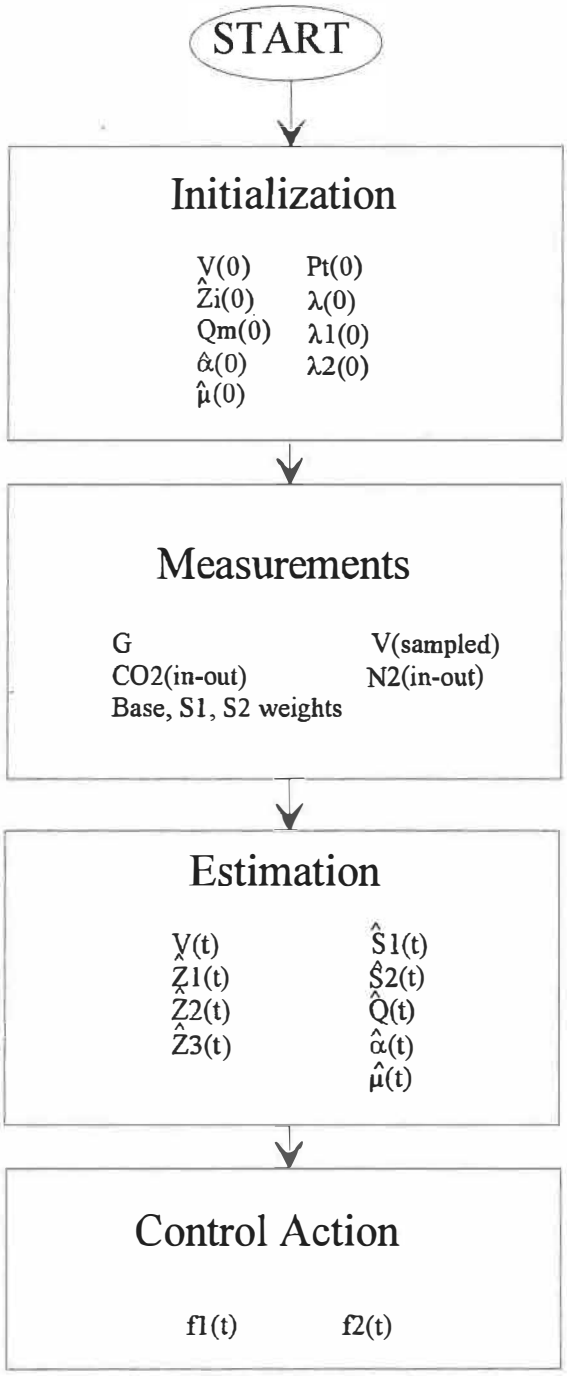

Figure 4.2 The flowchart of the control program written in Turbo Pascal

Table 4.6 Initial values of the tuning parameters used in the control program

Parameter	Initial value
CO_2 transfer rate per volume, Qm(0)	calculated as (CTR/V) at time zero
$\alpha(0)$, Eq (3.29)	the same as $\mu(0)$
The specific growth rate, $\mu(0)$	0.07 (h^{-1})
Pt(0), Eq (3.30)	200
Forgetting factor, Eq (3.30), λ	0.80
Glucose control law tuning parameter, $\lambda 1(0)$	0.80 * (1/T)[1]
Ammonium control law tuning parameter, $\lambda 2(0)$	0.80 * (1/T)

4.4 Analytical methods

4.4.1 Processing of fermentation samples

At each sampling time, about 30 mL of the culture broth was taken for analysis. After measuring the pH and the optical density (OD_{650}) of the sample, 2X5 mL were used to measure biomass dry weight (CDW), 2X2 mL for PHB and 2X2 mL for protein concentrations. Each tube was centrifuged at 7000 RPM for 10 minutes and then decanted. The supernatant was used for glucose and ammonium analysis.

For optical density measurement samples were diluted in 0.9% saline solution to give an OD between 0.1 and 0.6 at 650 nm. The spectrophotometer used was a Sequoia-Turner model 390.

[1]T: sampling period in hour

4.4.2 Biomass dry weight and cellular protein

Two tubes of the culture broth, each five mL, were centrifuged for 10 min at 7000 rpm at about 4°C in a Beckman J2-21 centrifuge. Their supernatants were decanted and frozen for further analysis. The pellets were resuspended in 5 mL distilled water and recentrifuged. Their supernatants were discarded and the pellets were again resuspended in 5 mL distilled water. The contents of each centrifuge tube were transferred to a preweighed aluminum dish and dried at 105°C for 24h to a constant weight giving the dry biomass. Then the aluminium dish was cooled for 5 to 10 min in a desiccator before being weighed.

For protein measurements two tubes each 2 mL of culture broth were centrifuged. The pellet was washed with distilled water, recentrifuged, and analyzed for protein content by the biuret reaction [Stickland 1951] with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard.

4.4.3 Glucose determination

The glucose content of the samples was determined by measuring the absorbance at 660 nm after a reaction with 3,5-dinitrosalicylic acid according to Miller's method [Miller 1959]. Standards of 0-2 (g/L) glucose were prepared. Their absorbances were linearly correlated to their concentrations. The concentration of the samples were related to their absorbances through the regressed line. The OD reading was always between 0.1 and 0.6.

4.4.4 Ammonium ion determination

Ammonium ion concentration was measured using an ammonium probe (Orion Reasearch Inc., Cambridge, Mass.) connected to a voltameter which gave mV readings. Standards had concentrations between 10 to 1000 ppm. The mV readings were used to obtain a linear standard line of the general form: $\ln(S_2)=a(\mathrm{mV})+b$.

4.4.5 PHB determination

PHB was measured from the frozen biomass pellets. PHB samples were prepared by the method of Riis and Mai method [Riis *et. al.* 1988]. The resulting propyl esters of the PHB monomers were quantified by gas chromatography. The helium flow rate through the 25-m-long HP5 capillary column (Hewlette-Packard Co., Palo Alto, Calif.) was 30 mL min^{-1}. The flame ionization detector was supplied with 500 mL of air, 30 mL of hydrogen, 30 mL of auxiliary helium min^{-1}. Injections of 1-μL were made. The injection port temperature was 180°C, and the detector temperature was 200°C. The initial oven temperature was 120°C (maintained for 1 min), increasing by 8°C min^{-1} to a final temperature of 210°C. The internal standard benzoic acid, and the external standard was a P(HB-co-HV) copolymer (Biopol, Zeneca).

4.4.6 Off gas analysis

A mass spectrometer (VG Quadrupoles) was used to analyze the air the exit of the fermentor and compare it with the air input to the fermentor. The percentages N_2, O_2, CO_2, and Ar, were calculated by the resident software, Spectralab PC.

Chapter 5: Results and Discussion

In this chapter experimental results obtained from the four fermentations described in Chapter 4 are presented. The information gathered from each of the first three fermentations formed the basis for decisions regarding each subsequent fermentation. Since the estimation algorithms developed in Section 3.4 are only applicable when certain assumptions are valid and certain parameters are known, data from the first three fermentations were employed to verify the assumptions and to calculate the required values.

In Section 5.1 the reaction network finally used in the state estimator is presented. In the following sections the values of CO_2 and substrate yields are calculated off-line and compared to the values found in literature, Sections 5.2 and 5.3. Since the efficient estimation of biomass from CO_2 off gas analysis data was necessary to justify the choice of the measurable variable, this issue is addressed next, in Section 5.4. In Section 5.5 the performance of several estimation methods of μ are examined. To conclude this chapter, the results of the last fermentation are examined and discussed.

5.1 PHB content of biomass and its effect on yield coefficients and the estimation algorithm

Significant quantities of PHB were produced in the growth phase of all three controlled fermentations in the presence of NH_4^+ (Figures 5.1 to 5.3), see Appendix III. In no experiment did the NH_4^+ concentration drop below 0.5 g/L. PHB had not been expected to account for more than 20% of the total biomass during the growth phase [Dochain *et. al.* 1989, Kim 1994, Ramsay *et. al.* 1990c].

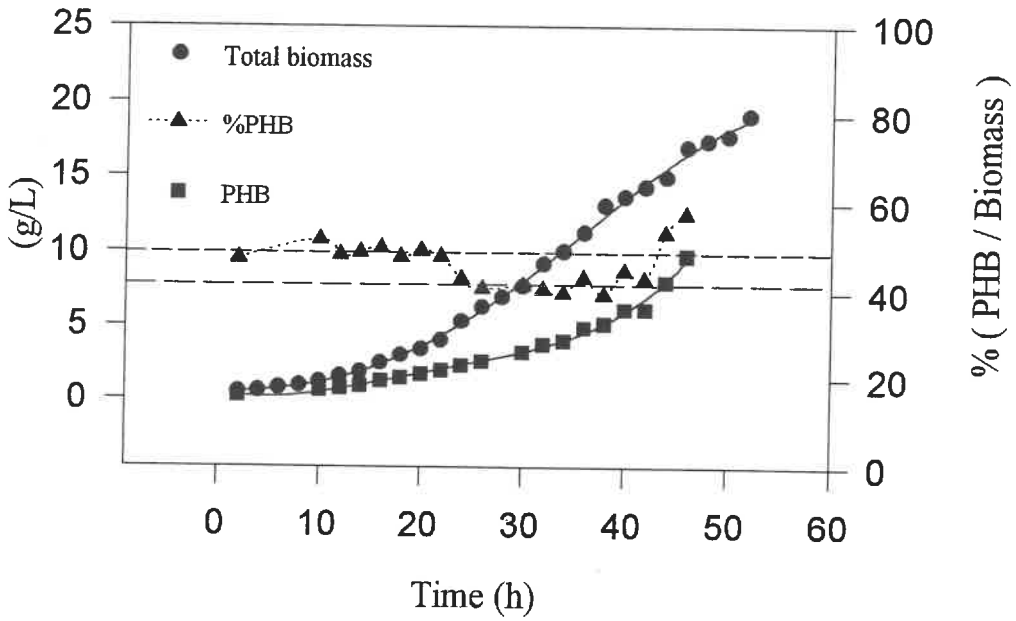

Figure 5.1 PHB content of biomass. Data from Fermentation CI

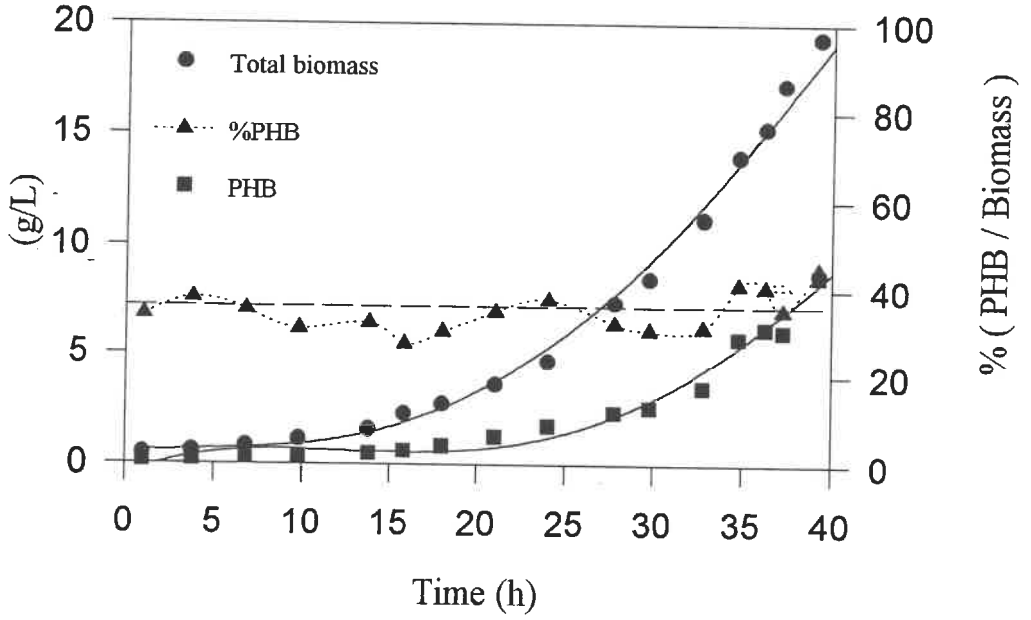

Figure 5.2 PHB content of biomass. Data from Fermentation CII

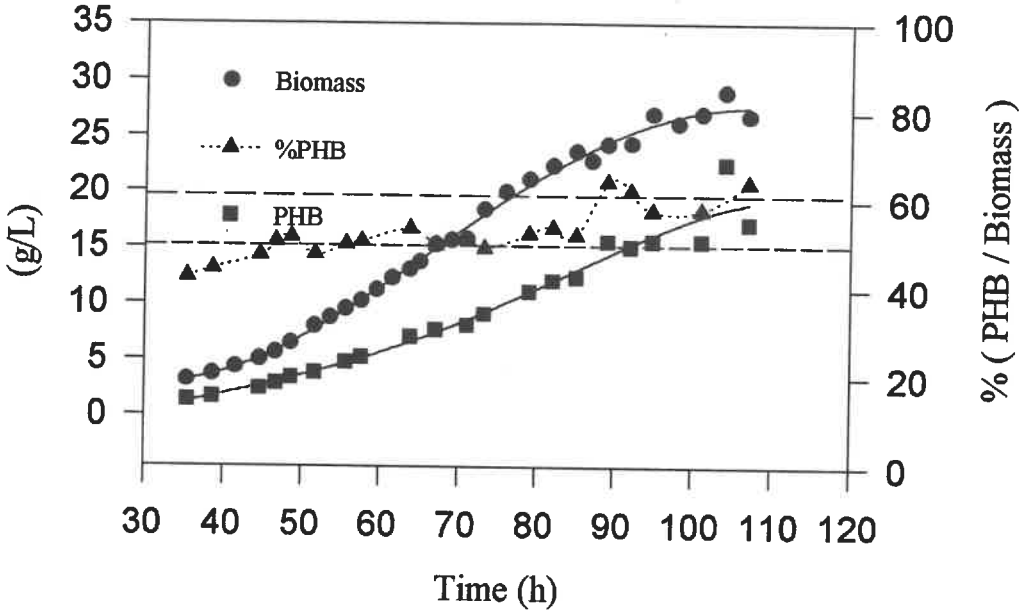

Figure 5.3 PHB content of biomass. Data from Fermentation CIII

This phenomenon conflicted with the selected reaction network (Eq 2.1 and 2.2). Neither the state estimator nor the yield coefficients were still valid. The solution was either to abandon the developed algorithms or to adjust them according to this new information.

The reaction network was modified accordingly. Since only the growth phase was studied, growth and PHB production can be seen as two parallel reactions. PHB production uses very little nitrogen. Thus when growth and PHB production occurs simultaneously the new reaction network can be presented as:

$$O_2 + \text{glucose} \begin{cases} + \; NH_4^+ \longrightarrow \text{biomass} + CO_2 \\ \longrightarrow \text{PHB} + CO_2 \end{cases} \qquad (5.1)$$

The influence of having both reactions occurring simultaneously must be reflected in the consumption of nutrients and their apparent yields. Based on the fact that both PHB production and growth produce carbon dioxide, the use of the CO_2 transfer rate as the measured variable for state estimation was still possible.

In the new reaction network (Eq 5.1) CO_2 is produced in both reactions and consequently possesses two different yield coefficients with regard to each one. When there is formation of a growth-associated product such as PHB, the calculation of yield coefficients needs more attention because carbon source is used in two reactions, growth and product formation. Hong [1989] has discussed this possibility and concluded that when there was growth-associated product formation, experimental determination of yield coefficients was not straightforward, and the fraction of carbon source consumed

for growth and product formation should be known *a priori*. If this fraction is constant the reaction rates are proportional, and each component has a constant yield in each reaction. Therefore the apparent (global) yield values can be calculated which are also constant.

As can be seen from Figures 5.1 to 5.3, during each individual fermentation this fraction was rather constant provided no drastic change in substrate concentrations occurred. Therefore both reactions could be combined and the apparent yields used in the estimator. Because the structure of the global reaction (Eq 5.2) is similar to Eq 2.3, the same estimator can be used for this global reaction.

The global reaction according to Eq 5.1, comprises both growth and PHB production:

$$k_{(O_2)}\ O_2 + k_{(S1)}\ S1 + k_{(S2)}\ S2 \Rightarrow X_{tot} + k_{(CO_2)}\ CO_2 \tag{5.2}$$

The apparent yields of CO_2, glucose, and NH_4^+ from biomass can be calculated according to the global reaction Eq 5.2. For CO_2 it can be readily shown that with γ as the ratio of PHB to active biomass (X_a), the global (apparent) k_{CO2} becomes:

$$\{k_{CO_2}\}_{global} = \frac{k_{CO_2/X_a} + k_{CO_2/P_2} \cdot \gamma}{(1+\gamma)} \tag{5.3}$$

where:

X_a (active biomass) = X_{tot} (total biomass) + $P2$ (PHB),

$k_{CO2/Xa}$ is the yield of CO_2 from the active biomass (growth reaction),

$k_{CO2/P2}$ is the yield of CO_2 for the PHB production reaction,

$\{k_{CO2}\}_{global}$ is the apparent (global) yield of CO_2 considering a global reaction.

Following the same reasoning the apparent yield of glucose per unit of total biomass can be presented as:

$$\{k_{S_1}\}_{global} = \frac{k_{S_1/X_a} + k_{S_1/P_2} \cdot \gamma}{(1+\gamma)} \tag{5.4}$$

For NH_4^+ there is no contribution from PHB production reaction , therefore the apparent yield becomes:

$$\{k_{S_2}\}_{global} = \frac{k_{S_2/X_a}}{(1+\gamma)} \tag{5.5}$$

It is evident that if the PHB-biomass fraction and the other yield coefficients are constant, the apparent yields are also constant. This additional information validates the use of the developed algorithms, regardless the production of PHB.

Bastin *et. al.* [1992] investigated identification problem for a reaction network where growth and production are decoupled. They demonstrated that if decoupling between growth and production was an erroneous assumption, and the two reactions (growth and

product formation) occurred simultaneously but with proportional reaction rates, these two reactions would be indistinguishable, and could be combined into a global reaction with apparent (global) yield coefficients. The expressions they obtained for global yield coefficients are similar to those presented above.

It is, however, important to comment on the effect that variation in the PHB-biomass fraction could exercise on the estimator. It might be inferred that if this fraction changes, the estimator will totally fail. But the following parameter sensitivity analysis shows that even 10% variation in γ results in less than 4% variation in the apparent yield coefficient.

The relative parametric sensitivity (RPS) is defined in Eq 5.6 where f stands for the function and P for the parameter (Volesky *et. al.* 1992).

$$\textit{Relative Parametric Sensitivity} = \left|\frac{P\partial f}{f\partial P}\right| \approx \left|\frac{P\Delta f}{f\Delta P}\right| \tag{5.6}$$

The sensitivity of the glucose apparent yield to the PHB-biomass fraction, γ, is calculated as shown in Eq 5.7. As for the CO_2, it manifests the same sensitivity as that of glucose without the second fraction (Eq 5.7).

$$\begin{aligned} \textit{RPS (Glucose or } NH_4^+ \textit{ Apparent Yield)} &= \left| \frac{\gamma}{(1+\gamma)} \cdot \frac{(k_{Si/P_2} - k_{Si/X_a})}{(k_{Si/X_a} + k_{Si/P_2}\gamma)} \right| \\ \textit{RPS } (CO_2) &= \left| \frac{\gamma}{(1+\gamma)} \right| \end{aligned} \tag{5.7}$$

If γ is perturbated, $\pm\delta$, the percentage of variation in the apparent yield is calculated similar to the RPS:

$$\textit{Relative Perturbation in Apparent Yields } (S_1, S_2) = \left| \frac{\sigma}{(1+\gamma)} \cdot \frac{(k_{Si/P_2} - k_{Si/X_a})}{(k_{Si/X_a} + k_{Si/P_2}\gamma)} \right|$$

$$\textit{Relative Perturbation in Apparent Yield } (CO_2) = \left| \frac{\sigma}{(1+\gamma)} \right| \tag{5.8}$$

Thus $+10\%$ perturbation in a $\gamma = 0.5$ results in less than ($0.05/(1+0.5) \approx 0.033$) or 3.3 % perturbation on the apparent yields of glucose and ammonium, because the second quotient on the right hand side of Eq 5.8 is smaller than unity, and 3.3% perturbation on the apparent yield of CO_2. Therefore even if the PHB-biomass ratio is perturbated during the fermentation, it is much less reflected on yield coefficients.

5.2 Carbon dioxide yield coefficient

The state estimation algorithm is based on known and constant CO_2, glucose, and ammonia yield coefficients. Literature data and the data obtained during this work were compared, and the results are presented in this section and the following. Several CO_2 yield coefficient values for the growth phase of *Alcaligene eutrophus* have already been reported. There are 0.93 and 1.03 [Lefebvre 1992], and 0.76 [Kim *et. al.* 1994] $g(CO_2)/g(\text{total biomass})$. The carbon source used was glucose except for 1.03 data where lactic acid was the carbon source.

The details of yield coefficient calculation from fed-batch fermentation data of this work have been explained previously [Cornet 1993]. The equation used to calculate CO_2 yield coefficient, which can be readily obtained by integrating Equation 3.4 (dynamics of CO_2) assuming a constant yield, is:

$$k_{CO_2/X} = \frac{\Delta[CO_2]_{liq} + \int_{t_0}^{t_f} CTR \cdot dt + \int_{t_0}^{t_f} [CO_2]_{liq} \cdot D \cdot dt}{\Delta(X) + \int_{t_0}^{t_f} D \; X \; dt} \tag{5.9}$$

This equation results in the cumulative apparent yield coefficient in the case of PHB production. The CO_2 concentration in the liquid phase, $[CO_2]_{liq}$, is negligible when compared to the other terms. Therefore it was speculated that elimination of those terms containing $[CO_2]_{liq}$ would result in a workable but much simpler equation as shown below.

$$k_{CO_2/X} = \frac{\int_{t_0}^{t_f} CTR \cdot V \cdot dt}{\Delta(X \cdot V)} \tag{5.10}$$

As can be seen in Figure 5.4, the effect of the neglected terms in the $k_{CO2/X}$ calculation is negligible after 10 h. The calculated CO_2 yield for each of the four fermentations is summarized in the Table 5.1. This parameter varied between each fermentation, yet its comulative value was constant during each fermentation. This variation could be due

to experimental error in air flow rate measurement and mass spectrometer analysis or it could be due to the variation in PHB-biomass ratio. The comulative CO_2 yield values during the four fermentations calculated at each sampling period are depicted in Figures 5.5 to 5.8.

Since both the growth of *A. eutrophus* and its production of PHB from glucose are associated with carbon dioxide production [Lefebvre 1992, Yamane 1993], the CO_2 transfer rate was used to monitor the state of the system. The asymptotic observer was the tool developed to estimate the biomass and substrate concentrations from CO_2.

Table 5.1 calculated CO_2 yield per unit of biomass for the conducted fermentations

Fermentation	kCO_2/X_{tot} [g(CO_2)/g(total biomass)]
Expo	0.78
CI	1.63
CII	1.28
CIII	1.13

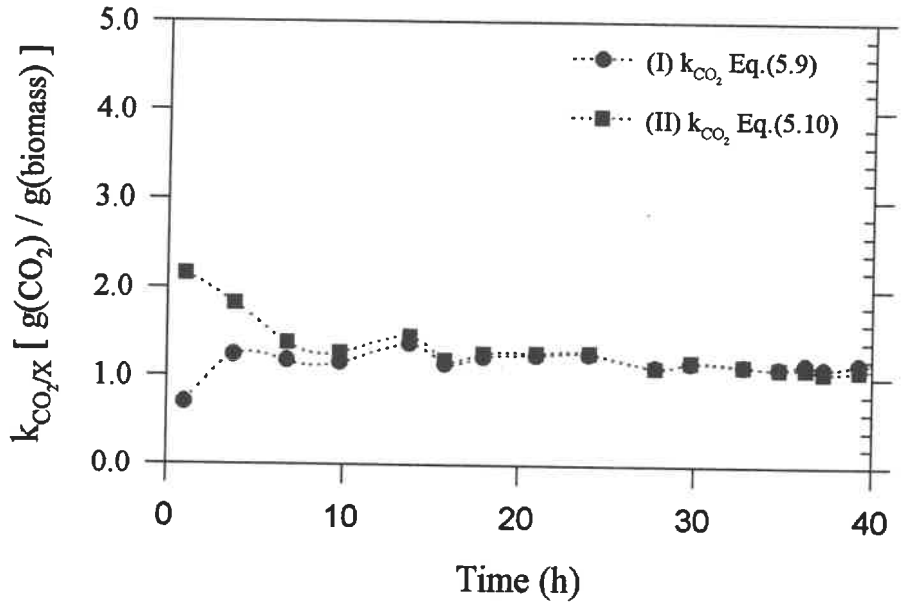

Figure 5.4 Comparison between 2 approximations for $k_{CO_2/X}$ calculation. Data from Fermentation CII

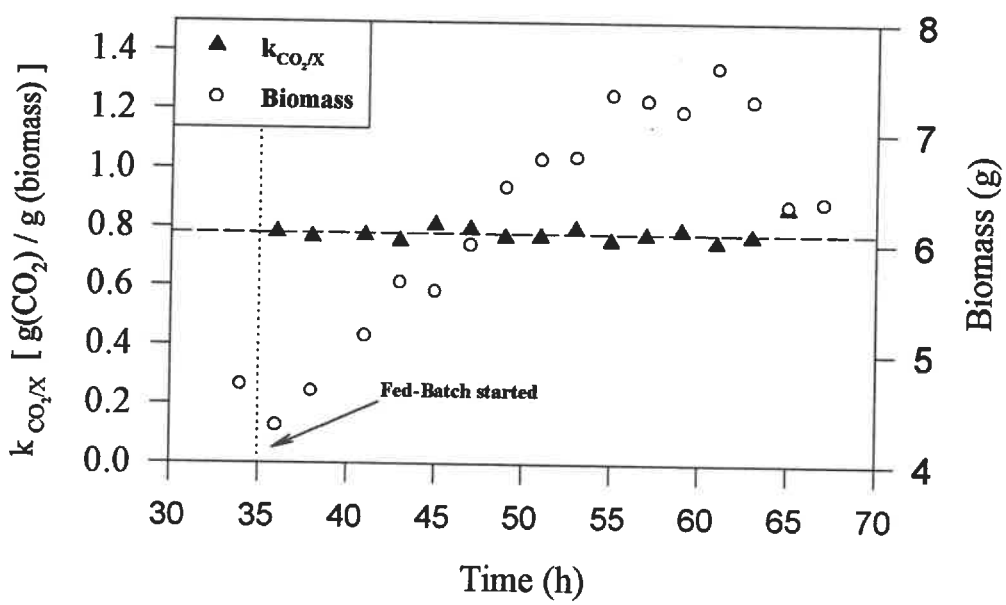

Figure 5.5 Biomass (CDW) and cumulative $k_{CO_2/X}$ (Fermentation Expo)

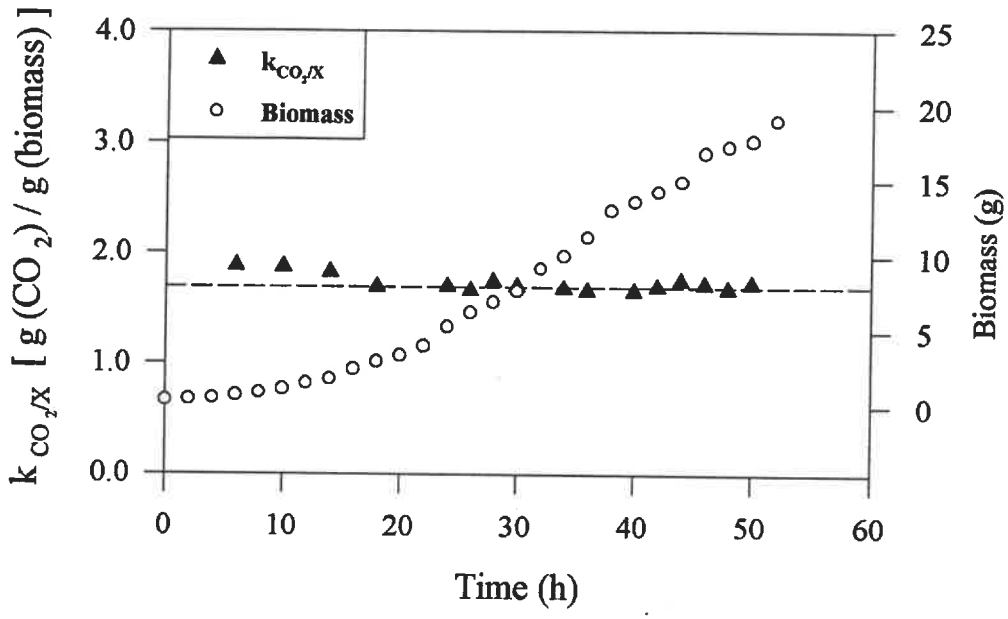

Figure 5.6 Biomass (CDW) and cumulative $k_{CO_2/X}$ (Fermentation CI)

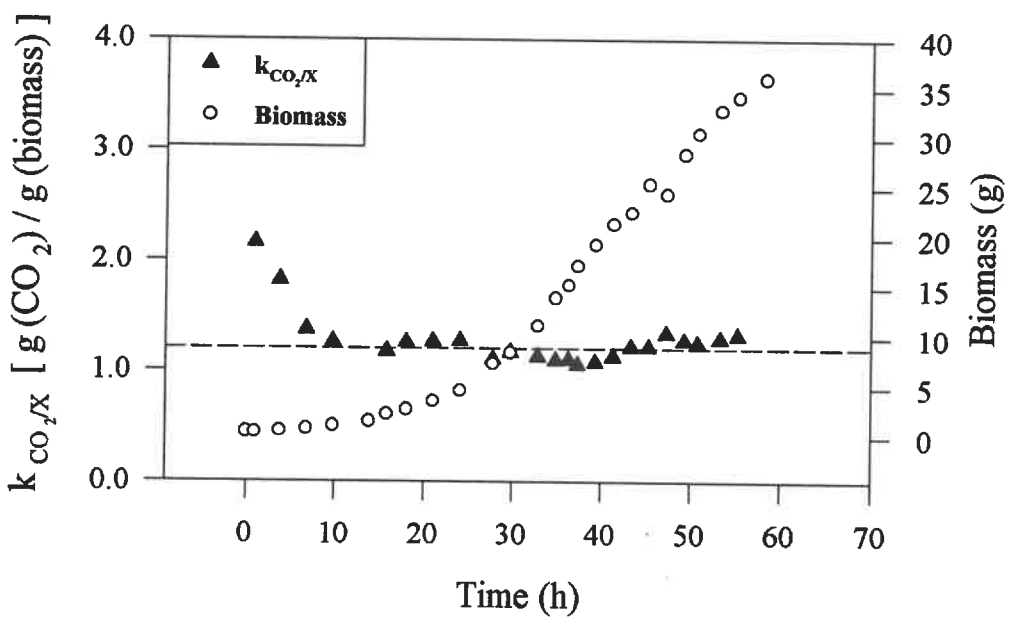

Figure 5.7 Biomass (CDW) and cumulative $k_{CO_2/X}$ (Fermentation CII)

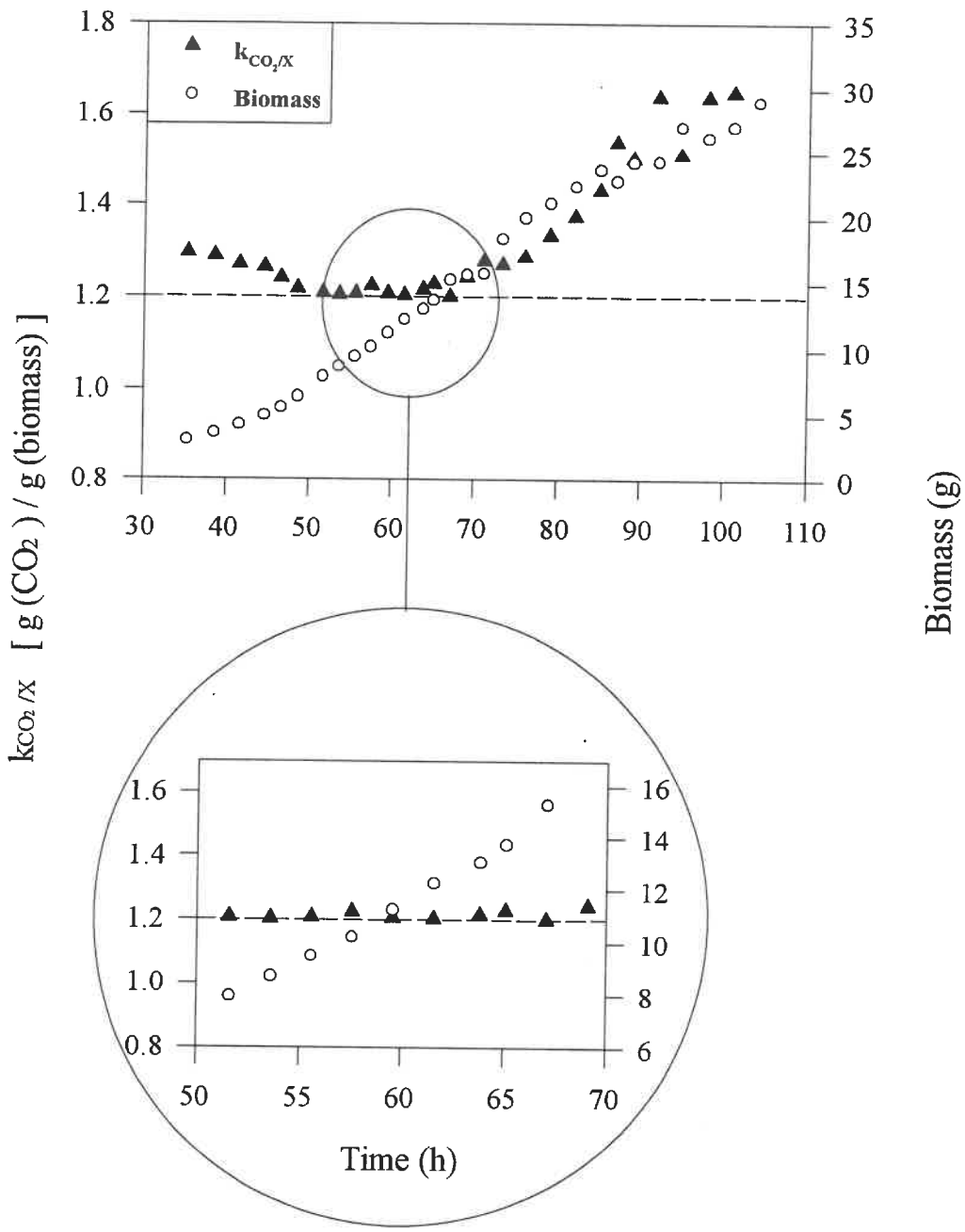

Figure 5.8 Biomass (CDW) and cumulative $k_{CO_2/X}$ (Fermentation CIII)

5.3 Relation between CO_2 concentration in the gas phase and growth

A fundamental premise of the proposed algorithms was the direct correlation between the carbon dioxide transfer rate and the microbial activity of the system. To verify this the asymptotic observer was applied to the off gas analysis data of experiments CI and CII.

As shown in Section 3.4, a prerequisite for biomass estimation from off gas data estimation was *a priori* knowledge of the yield coefficient and the carbon dioxide transfer rate data. In the estimation algorithm, a constant $k_{CO2/X}$ was used which differed for each fermentation. The $k_{CO2/X}$ used for the estimation was the commulative value calculated for the same fermentation, Table 5.1. In Figures 5.9 and 5.10 the estimated biomass from CO_2 is compared to Cell Dry Weight data (CDW). A good concordance existed between these two during the entire fermentation.

An important conclusion drawn from this work was the justification of a correlation between carbon dioxide transfer rate and microbial activity. The nearly perfect agreement between biomass estimation and CDW data confirmed that: *during each fermentation, biomass produced carbon dioxide with a constant yield* which implies that *it is possible to have an accurate estimation of biomass from off gas analysis data.*

5.4 Yield coefficient calculation for glucose and ammonium

The algorithm discussed in Section 3.4 is based on known and constant yield coefficients for CO_2, NH_4^+, and glucose. The next step towards the realization of a controlled fermentation was the evaluation of the glucose and NH_4^+ yield coefficients.

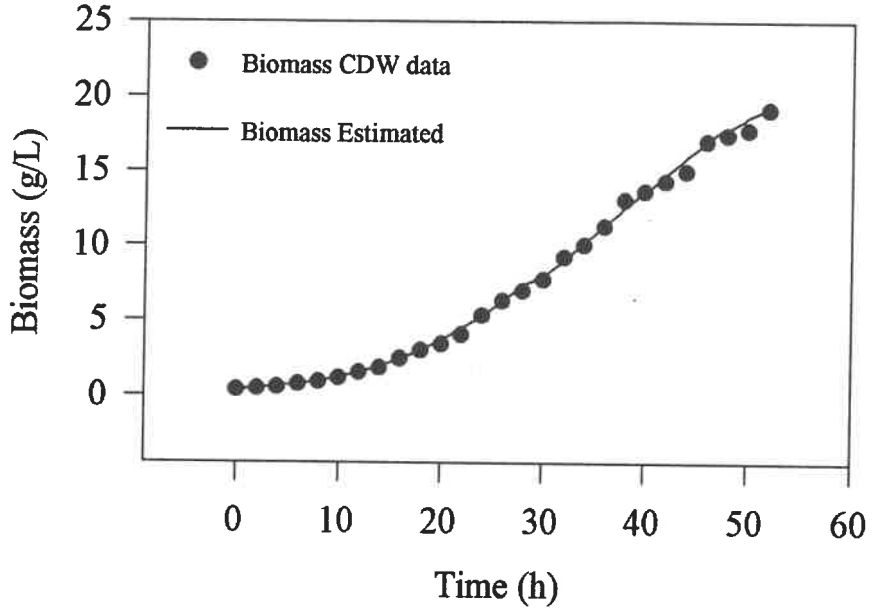

Figure 5.9 Comparison between estimated biomass and biomass dry weight. Data from Fermentation CI

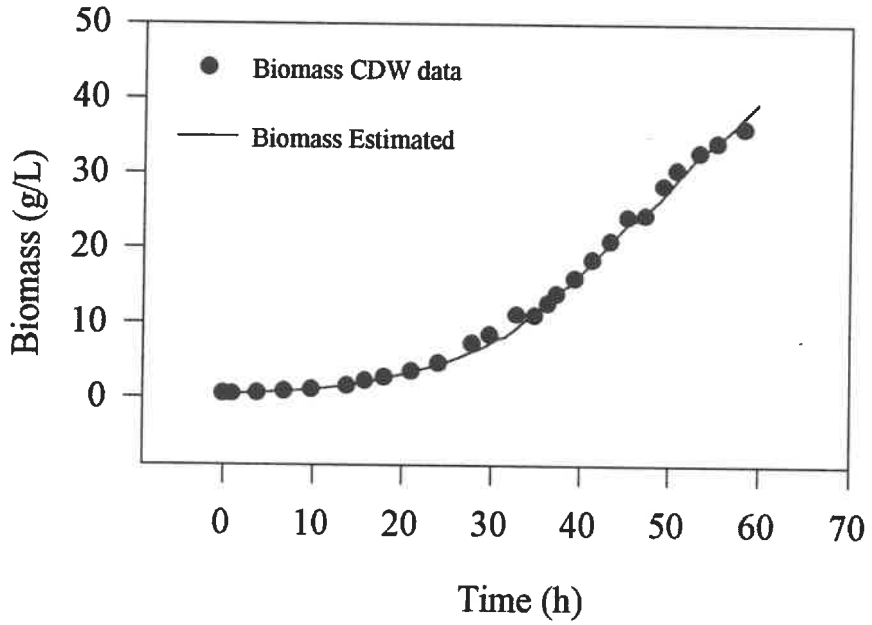

Figure 5.10 Comparison between estimated biomass and biomass dry weight. Data from Fermentation CII

Some of the available data for glucose and NH_4^+ yield coefficients during the growth phase of *A. eutrophus* from literature and previous work at École Polytechnique de Montréal are summarized in the Table 5.2.

Table 5.2 Some literature values for glucose and NH_4^+ yields

Source	$k_{glucose/Xtot}$	$k_{NH4+/Xtot}$
Ramsay (1990b)	2.70,2.12	0.17
Lefebvre (1992)	2.20	0.16
Seung (1994)	2.17	0.16
Lee (1991)	2.13	0.16
Mulchandani (1988)	2.17	0.19

The experimental data of experiment CII and CIII were exploited to determine the substrate yield coefficients. The equation used was [Cornet 1993]:

$$k_{S_i/X_{tot}} = \frac{-[\Delta(V \cdot S_i) - \int_{t_0}^{t_f} F_{i(in)} \cdot S_{i(in)} \cdot dt]}{\Delta(V \cdot X_{tot})} \tag{5.11}$$

The calculated yields for both glucose and NH_4^+ at each sampling time for the experiments CII and CIII are depicted in Figure 5.11, and 5.12. The large variation seen in data from early in fermentation data is due to the sensitivity of calculation to biomass data. At the beginning biomass concentrations are low, so any error becomes magnified in the denominator of Eq 5.11.

5.5 Performance of the specific growth rate estimation methods

The specific growth rate was the only parameter to be estimated in the control law. In Section 3.5 three different algorithms were presented. There were an observer based algorithm with two methods of tuning, a recursive least squares, and a material balance estimation. Here these methods are assessed using the data from the fermentation CI.

In Figure 5.13 the first two methods are applied to the same data. The results are compared to the off-line estimation from cell dry weight data using cubic spline smoothing. The cubic spline method is not highly accurate but rather it gives a range within which the data points should lie. As it can be seen, the results of these methods fall inside this interval, which ensures accurate estimation. Although these three methods are different, the differences in the results they produce are relatively small.

An estimation based on a direct material balance (Section 3.5.4) was also applied to the same series of data. Its result was compared to least squares method result, Figure 5.14. Also the estimates of μ by the cubic spline method were used to present a confidence interval for comparison. The error in CTR data was more prominent at the beginning of fermentation because CO_2 transfer was low which resulted in poor μ estimates for this period when the direct material balance was used. The recursive least squares method was chosen because it minimises the error and does not require the CO_2

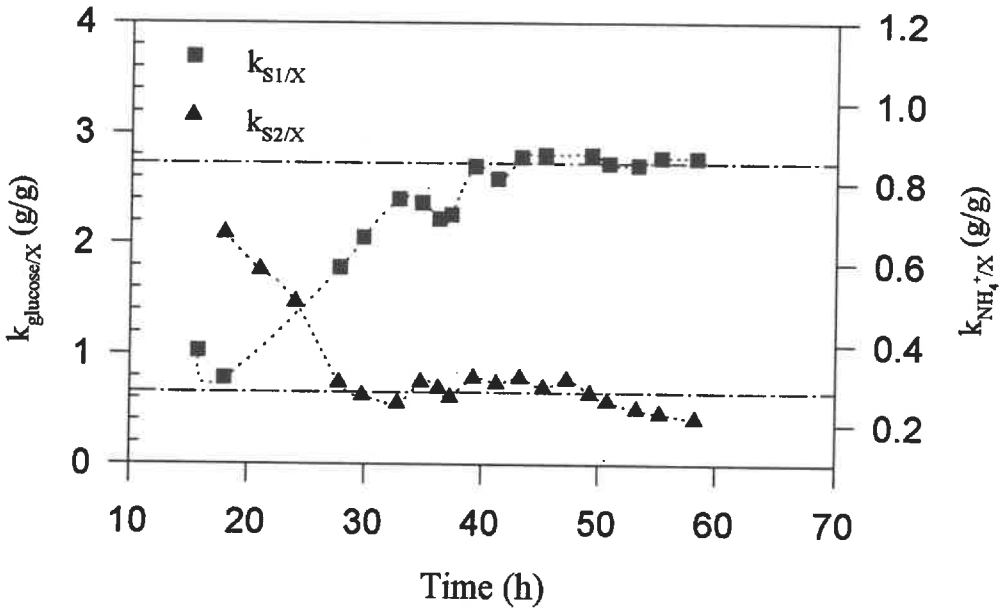

Figure 5.11 Glucose and NH_4^+ cumulative yield coefficients (Fermentation CII)

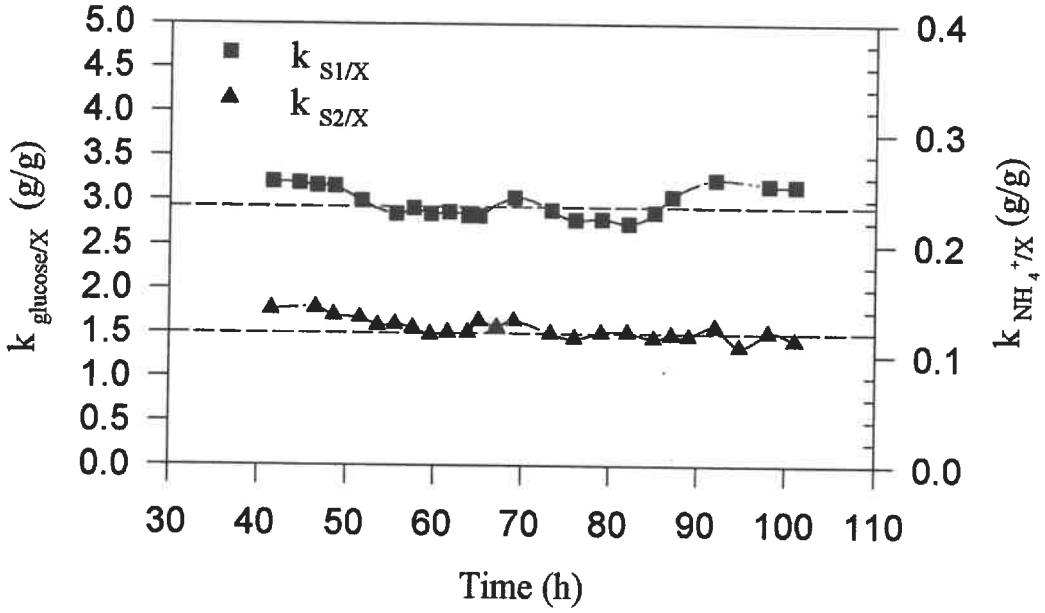

Figure 5.12 Glucose and NH_4^+ cumulative yield coefficients (Fermentation CIII)

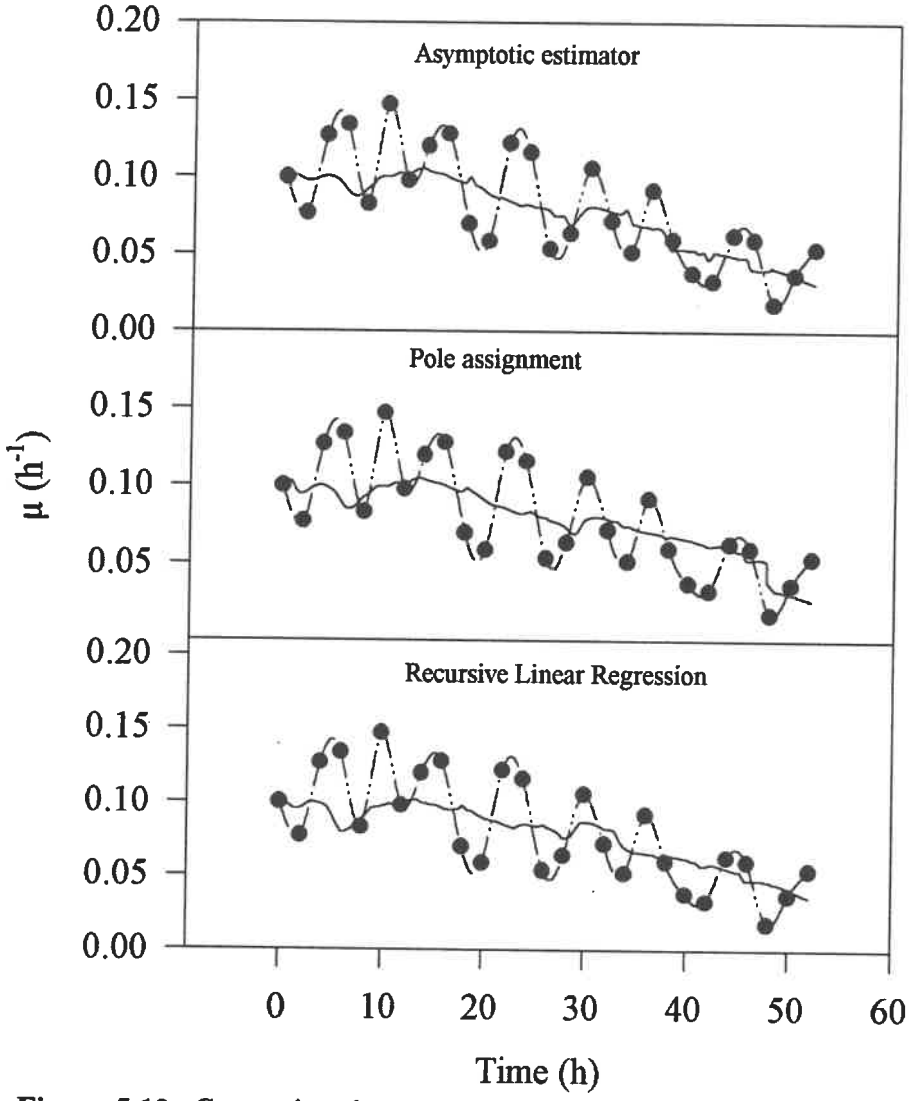

Figure 5.13 Comparison between the three proposed μ estimation methods and estimated μ from biomass dry weight data by cubic spline method

—— μ estimated by observer-based estimator (asymptotic and pole assignment) and least squares method

—●·- μ from biomass dry weight data, the cubic spline

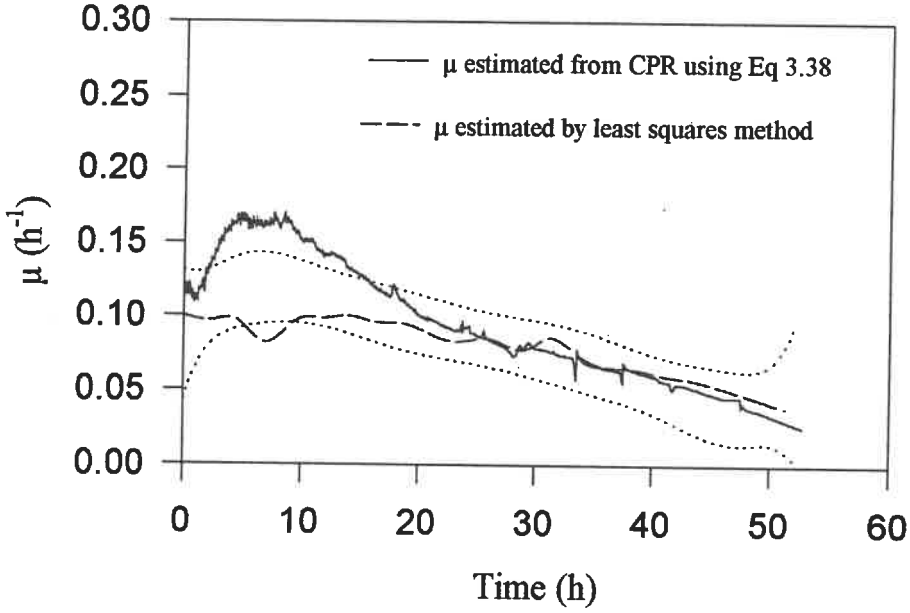

Figure 5.14 Comparison between estimation of μ from CPR data of Fermentation CI. Mass balance method (Eq 3.38) and least squares method (Eq 3.29). Broken lines are the 95% confidence interval when cubic spline off-line estimates of μ are fitted to a 5th order polynomial.

yield coefficient in contrary to the material balance method.

5.6 Fermentation CIII

Fermentation CIII was carried out with adaptive control applying the asymptotic observer for biomass and both substrates, and specific growth rate estimation and a completed control program. The different periods of this fermentation are referred to by the letters *a-h* as presented in Figure 5.15. Because of the uncertainty about the percentage of PHB in the biomass , samples from the beginning and the end of the batch phase (period *a*) were analyzed. The yield coefficients for CO_2, glucose, and NH_4^+ were calculated for this phase. They were 1.13 g(CO_2), 2.14 g(glucose), and 0.12 g(NH_4^+) per gram of total biomass where biomass was estimated from optical density. The second phase (period *b*) continued until 53.6 hours when a sample was analyzed for glucose and NH_4^+. The results indicated that NH_4^+ was well controlled (1.97 g/L) but that the glucose concentration had decreased to 8.8 g/L. This indicated that the yield coefficients used in the control algorithm were accurate for CO_2 and ammonium, but not for glucose. Therefore the measured glucose concentration and a higher glucose yield coefficient were entered into the control program (8.8 g/L and 2.5 g(glucose)/g(biomass) respectively). The statistical report for the period *b-e* is tabulated in Table 5.3. The glucose and NH_4^+ concentration set points for this period were 15 g/L and 2 g/L respectively.

Table 5.3 Statistical parameters of measured glucose (period *c-e*) and ammonium (period *b-e*) concentrations (Fermentation CIII)

Statistical Parameter	Glucose (g/L)	Ammonia (g(NH_4^+) /L)
mean	15.10	1.97
standard deviation	0.56	0.06
minimum	14.26	1.87
maximum	15.90	2.10

The controller glucose yield was not changed again until the end of the experiment. However various technical problems occurred which affected the general performance of the control. A chronological description of those events based on Figures 5.15 and 5.16 now follows.

At 73.35h, glucose and ammonium were analyzed. The results revealed that ammonium was still under good control while glucose has increased to 17.2 (g/L). The only change applied after this observation was the correction of the estimated glucose concentration in the control algorithm.

At the end of period *g*, glucose and ammonium were analyzed and because of their high concentrations compared to the set points, new set points were entered into the program. These were 17.5 g/L for glucose and 2.55 g/L for ammonium.

Another problem occured during period *f*. At the end of period *f* the control program was blocked for about 20 minutes due to the operator's error, therefore the substrate flow rates, normally calculated for the next 4 minutes, continued to flow for the entire 20 minute period. The curves showing substrate addition in Figures 5.15 and 5.16 shows this problem.

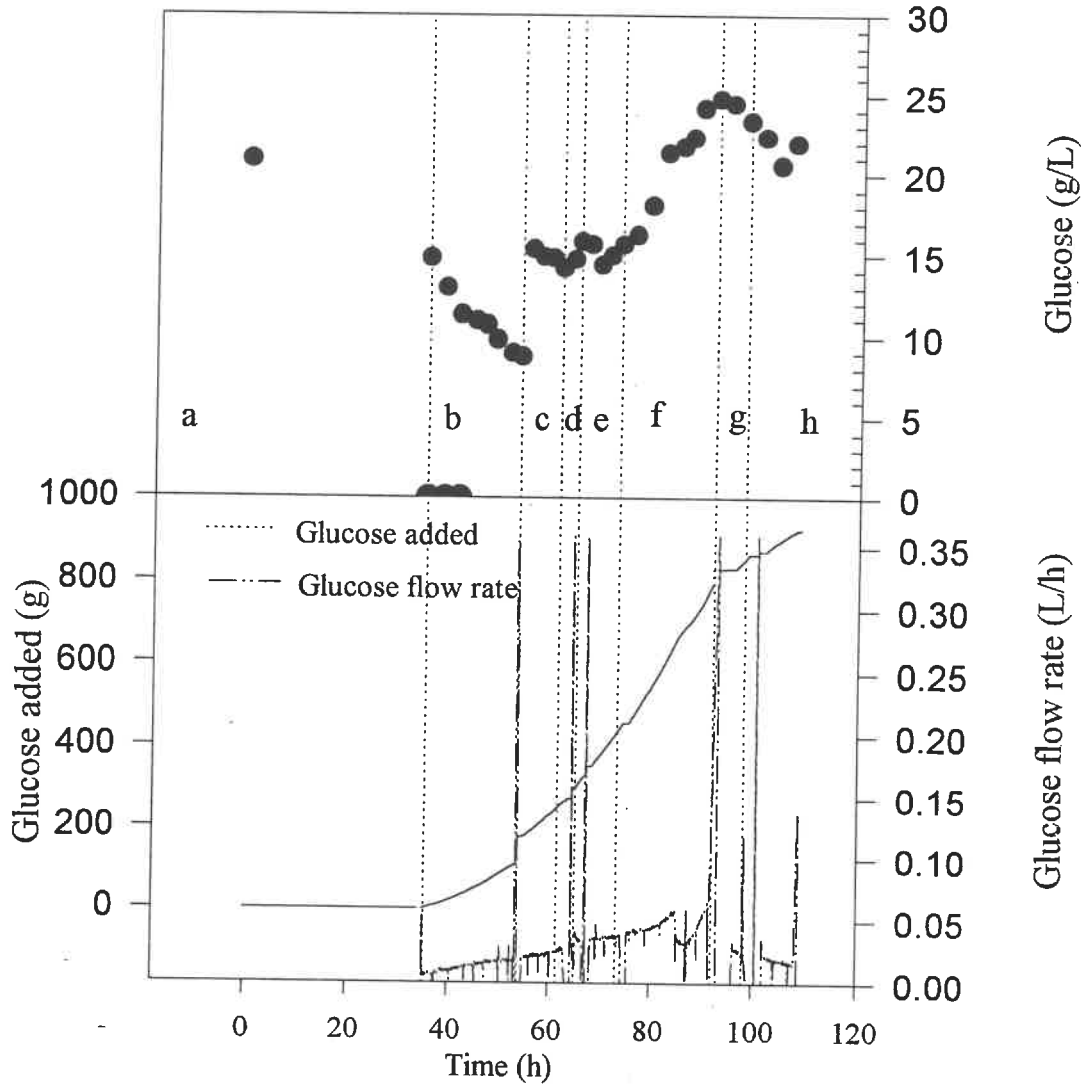

Figure 5.15 Glucose concentration, flow rate, and the amount added

(Fermentation CIII)

a : batch phase
b: first control phase, underestimation of glucose yield coefficient
c : second control phase, $k_{S_1/X}$ correction
d : problem with control program
e : control program blocked for a few minutes, air flow rate changed
f : air flow rate changed, S_1, S_2 correction
g: set point changes
h: fermentation end

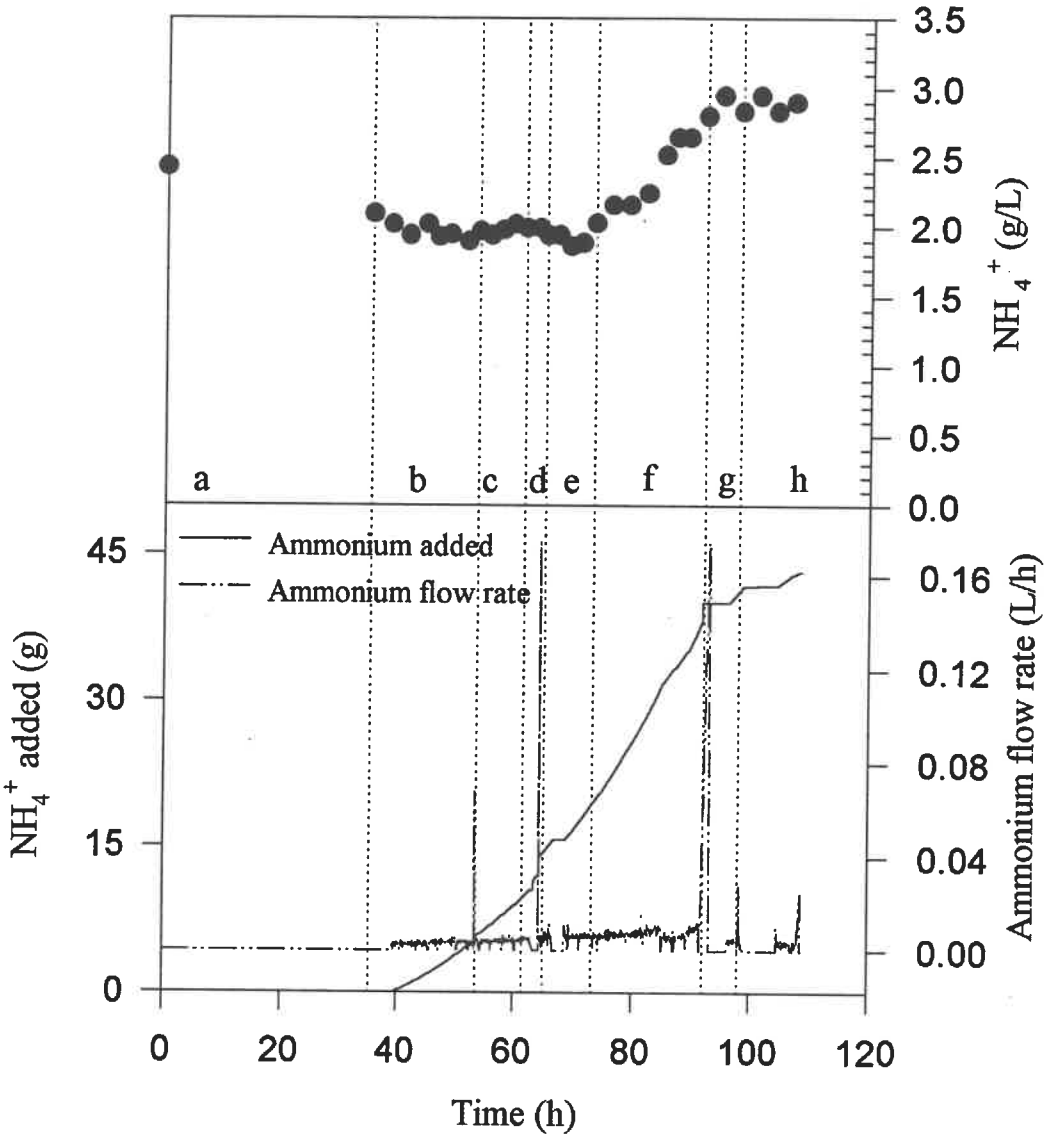

Figure 5.16 Ammonium concentration, flow rate and the amount added (Fermentation CIII)

As long as the dissolved oxygen probe was functioning the dissolved oxygen was always maintained above 30% of saturation by manipulating agitation and air flow rate manually. The exact moment of each of these manipulations can be seen in the CO_2 transfer rate (Figure 5.17). The break points correspond to changes. However at about 40 h the apparent dissolved oxygen concentration began to fluctuate between 40% and 120 %, due to a malfunction of the electrode cable. This problem lasted a few hours after which the dissolved oxygen readings were stable.

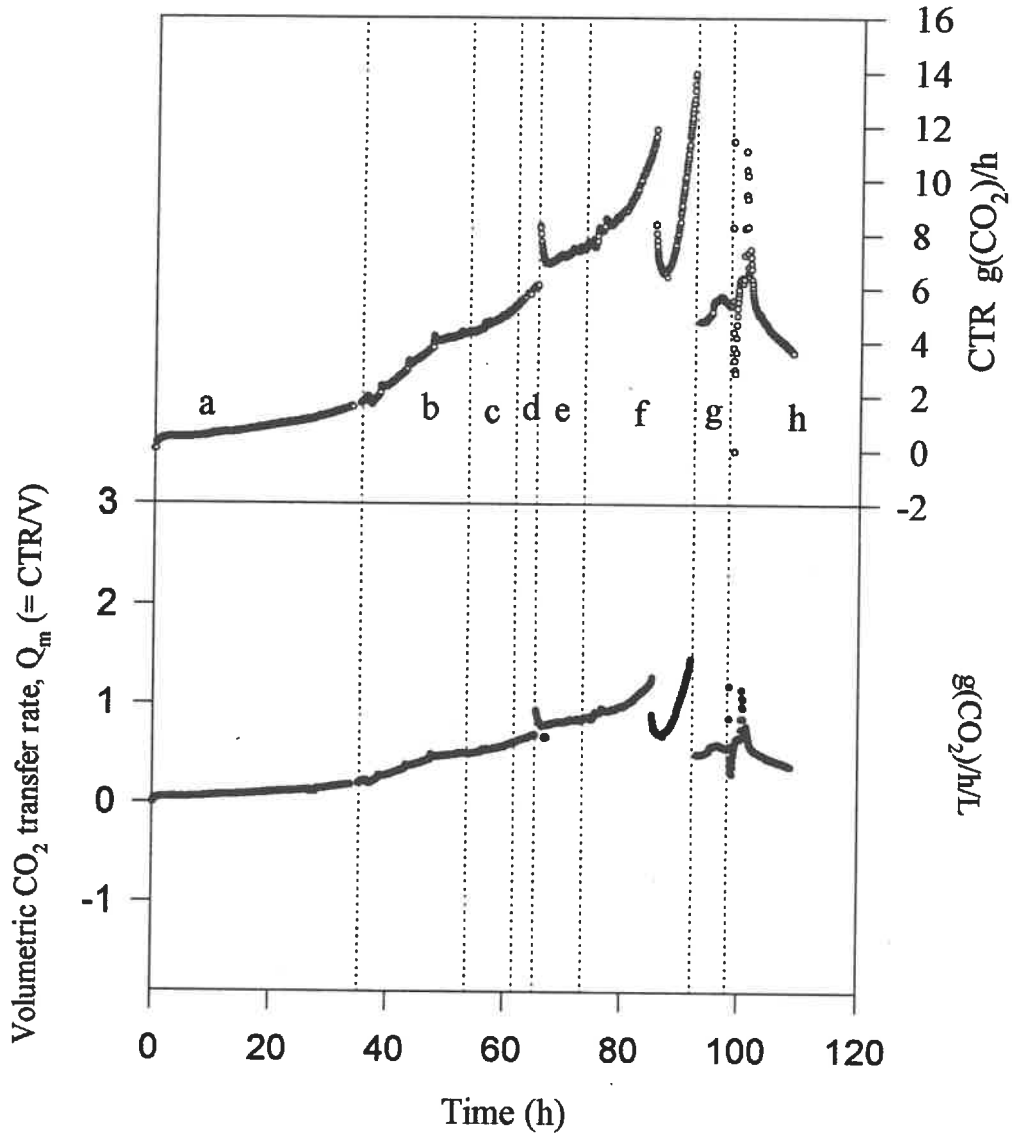

Figure 5.17 Volumetric CO_2 transfer rate (Q_m) and CTR data (Fermentation CIII)

5.7 Performance of the state observers and the controller

Since the controller used the estimated substrate concentrations, its performance depended on a good estimation. The values of yield coefficients (CO_2, glucose, ammonium) were also necessary to the state estimator. As explained in Section 5.6, the ammonium concentration was well controlled before the technical problem occurred (before 70 h), while glucose concentration was not well controlled during the period *b* due to use of the wrong yield coefficient in the estimation algorithm. However, after the period *b* glucose concentration was well controlled before the technical problem occurred (before 70 h). State estimates (asymptotic observer) and off-line data of biomass, glucose, and ammonium are depicted in Figures 5.18, 5.19, 5.20. Figure 5.18 indicates that biomass was well estimated until 70 h. After about 70 h both substrate concentrations increased.

The off-line estimation of μ, the carbon dioxide transfer rate data, and the behavior of the controlled variables (glucose and ammonium concentrations) were examined to find the reason of this malfunction. The biomass dry weight data were exploited to estimate specific growth rate. Estimation was performed by applying a similar observer based method explained in Appendix II, cubic spline method, and polynomial curve fitting. These estimates of μ were compared to those values used by the controller, Figure 5.21 and 5.22. Since the convergence rate of the observer based estimator depended on the sampling period, it was hard to determine when exactly the estimates and the actual values coincided. However, it depicted the trend of the specific growth rate changes. The other two methods showed that after about 70 h on-line estimation differed from off-line data. This could not be due to algorithm error, because it was proved to be convergent as it had been for the previous experiments as well as for the first 70 hours of Fermentation CIII.

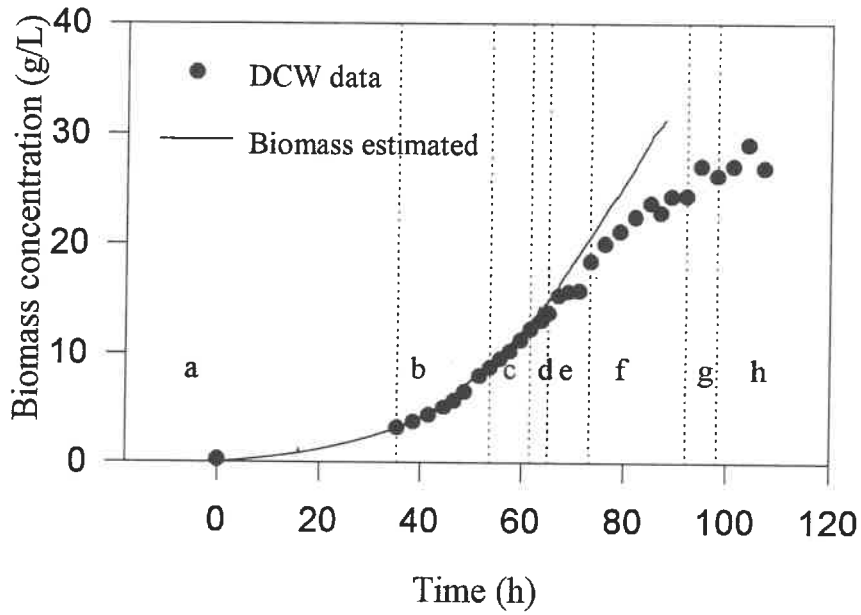

Figure 5.18 Biomass concentration, estimated and experimental. Data from Fermentation CIII.

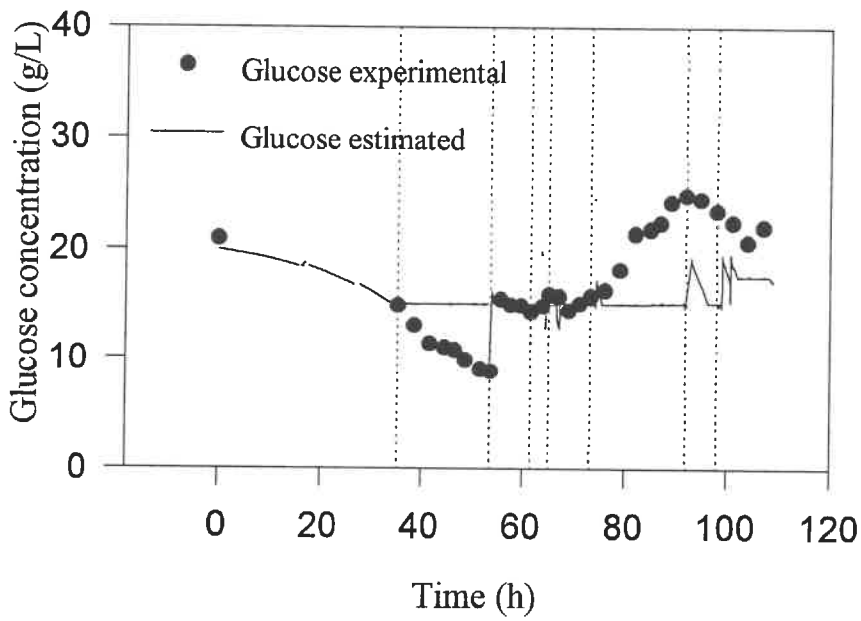

Figure 5.19 Glucose concentration, estimated and experimental

Data from fermentation CIII

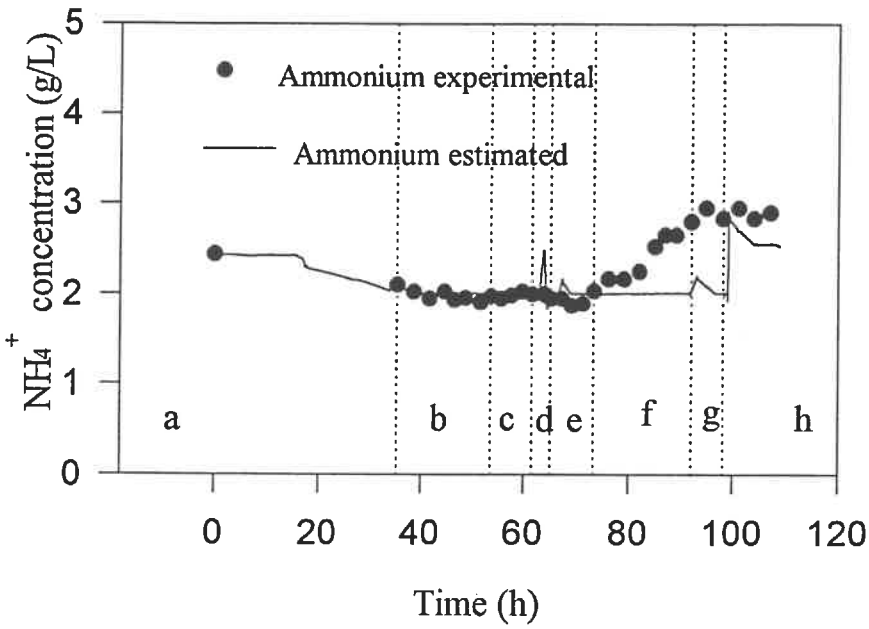

Figure 5.20 NH_4^+ concentration, estimated and experimental Data from Fermentation CIII

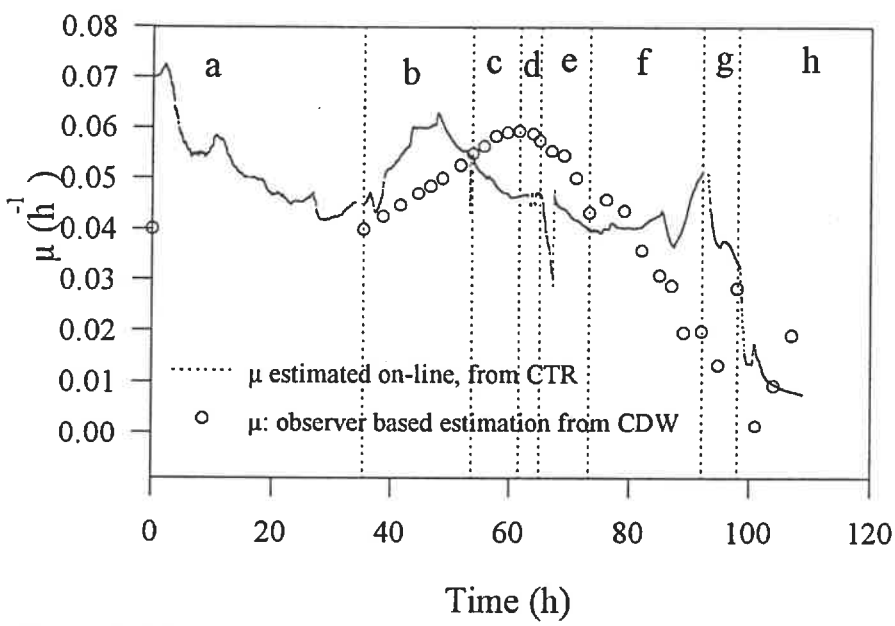

Figure 5.21 Estimation of the specific growth rate for Fermentation CIII.

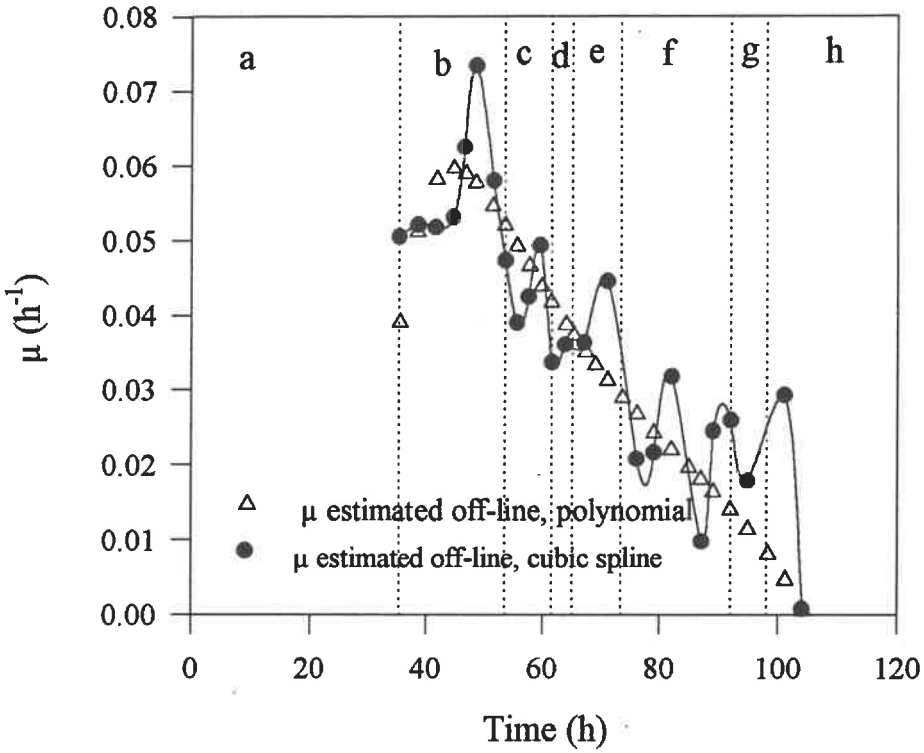

Figure 5.22 Estimation of the specific growth rate for Fermentation CIII.

The other verification was the estimation of $k_{CO2/X}$ using the same data available to the control program. Figure 5.8 shows an increase in $k_{CO2/X}$ after 70 hours while the air flow rate was not changed between 62.85 and 86.35 hours.

These phenomena, overerestimation of μ, increase in estimated $k_{CO2/X}$, increase of glucose and ammonium concentrations, overestimation of biomass, all indicated that from the control point of view there had been an increase in CO_2 transfer rate (CTR). An increase in CTR meant an increase in growth rate. A faster growth needed more substrate or an increase in substrate addition rate. However none of these corresponded to the real conditions. Biomass and specific growth rate were overestimated. Glucose and ammonium concentrations increased. Therefore the increase in CTR perceived by the control program was not real. The reason of this overestimation was incorrect air flow rate data used by the program. This was due to both measurement imprecision, and fluctuation in air line supply.

5.8 Concluding remark

The results of Fermentation CIII (Sections 5.6, 5.7) demonstrate that even in the presence of PHB production, the state estimator could result in accurate estimates provided the global yield coefficients are known and constant. During the periods *b-e* of fermentation CIII these prerequisites were met (Figures 5.8 and 5.12). Thus ammonium was well estimated and controlled and when accurate value of $k_{S1/X}$ was used, glucose was also well estimated and controlled. The fairly constant $k_{S1/X}$, $k_{S2/X}$, and $k_{CO2/X}$ for these periods allowed the state estimator to function well and accordingly there was good control (Figures 5.18 to 5.20). However, the technical problem resulting in overestimation of the CO_2 transfer rate, generated erroneous estimates in the substrate concentrations and the specific growth rate for the balance of the fermentation,

after approximately the 70 h mark.

Chapter 6: Conclusions and Recommendations

6.1 Conclusions

I) The assumption that there is little growth associated PHB production is not always valid. In the all the fed-batch cultures of this work, considerable PHB was produced during the growth phase.

II) The CO_2 cumulative yield coefficient was fairly constant, even though PHB production was high.

III) The biomass concentration and the specific growth rate were efficiently estimated from carbon dioxide transfer rate without need of the CO_2 yield coefficient.

IV) The PHB/biomass ratio was fairly constant throughout an individual fermentation. Therefore a global reaction could be used to replace the growth and production reactions in the asymptotic observer equation.

V) Constant substrate yield coefficients were obtained throughout each individual fermentation.

VI) Substrate concentrations (e.g. glucose and ammonium) can be simultaneously kept in an acceptable range around the set points

provided that certain hypotheses are met. If the reaction network corresponds to the actual fermentation, and yield coefficients are constant, the good simultaneous control of estimated glucose and ammonium concentrations is possible.

VII) If the produced PHB is not proportional to the total biomass, measurement of only the CO_2 transfer rate will not be adequate because the apparent yield coefficients are no longer constant and use of a global reaction network is not realistic.

6.2 Recommendations

I) A study should be conducted to determine the reasons for significant PHB production even when there is no nutrient limitation.

II) The air flow rate must be more precisely measured and controlled because the basis of the observer is the carbon dioxide transfer rate (CTR) calculated from mass spectrometer data and the air flow rate to the reactor.

III) For a fermentation where growth and product formation are not decoupled (reaction network 2.1 and 2.2), a set of at least two independent measurements is necessary for estimation purposes provided that the yield coefficients are known.

IV) If PHB and biomass concentrations were the measured variables,

there would be no need for the values of yield coefficients because the yield coefficients matrix (K) (Eq 3.4) would disappear when the model was transformed. Therefore, by measuring the biomass and PHB concentrations, the estimation algorithm would become robust and unaffected by uncertainty or variation in yield coefficient values or variation in the PHB/biomass ratio.

V) This controller could be applied to kinetic studies where there is double-substrate limitation, and to process optimization after the optimal concentrations of the substrates have been determined during the kinetic studies.

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APPENDIX I

CTR AND $[CO_2]_{LIQ}$ CALCULATION FROM OFF-GAS ANALYSIS DATA

In the formulation of the system dynamics (Eq 3.5) the variable assumed to be measured was the CO_2 concentration in the liquid phase, *P1*, but it is difficult to measure it accurately. This concentration is calculated from the available off-gas analysis assuming certain hypotheses which are presented in this appendix. The carbon dioxide transfer rate was also needed for the state and parameter estimation (Q_{CO2} term). The mass balance for the gas phase as well as the liquid phase now follows [Heinzle 1987].

a.1 Liquid phase[1]

$$V_l \frac{dc_{l,i}}{dt} = k_l a(c_{l,i}^* - c_{l,i})V_l + r_i X V_l \qquad \text{(a.1)}$$

a.2 Gas phase

$$V_g \frac{dc_{g,out}}{dt} = (\frac{M_i}{V_{tp}})(F_{in}x_{in,i} - F_{out}x_{out,i}) - k_l a(c_{l,i}^* - c_{l,i})V_l \qquad \text{(a.2)}$$

where:

V: volume,

c: concentration,

$k_l a$: volumetric mass transfer coefficient,

c^*: equilibrium concentration,

[1] Dilution effect or the transport of CO_2 with the liquid phase from the fermentor is neglected due to the low solubility of CO_2 in water.

r: reaction rate,

X: biomass,

x: mole fraction,

F: flow rate,

H: Henry coefficient.

M_i: the CO_2 molecular weight

V_{tp}: the CO_2 molar volume at the operating pressure and temperature

The indices are:

l: liquid phase,

g: gas phase,

i: component,

in: entrance to the reactor,

out: exit of the reactor.

The two concentrations are linked by Henry's law:

$$C_{l,i}^{*} = x_{out,i} H_i p_{tot} \tag{a.3}$$

if the gas phase is well mixed.

Since the fermentation under consideration is aerobic, the gas flow rate changes due to the consumption of oxygen and the production of carbon dioxide. Using the tie-elemnt concept for an inert gas, e.g. N_2, and assuming quasi steady-state conditions where the accumulation term is very small, the gas phase balance yields:

$$0 = F_{in} x_{in,inert} - F_{out} x_{out,inert} \tag{a.4}$$

Therefore, measuring the inlet flow rate, F_{in}, the exit mass flow can be estimated:

$$F_{out} = F_{in} \frac{x_{in,inert}}{x_{out,inert}} \tag{a.5}$$

Combining Equations a.1, a.2, and a.5 and assuming quasi steady-state conditions yield:

$$r_i X V_l = -F_{in} \left(x_{in,i} - x_{out,i} \frac{x_{in,inert}}{x_{out,inert}} \right) \cdot \left(\frac{M_i}{V_{tp}} \right) \tag{a.6}$$

CO_2 in the liquid phase enters an equilibrium reaction (because the time scale of changes in fermentation are sufficiently long):

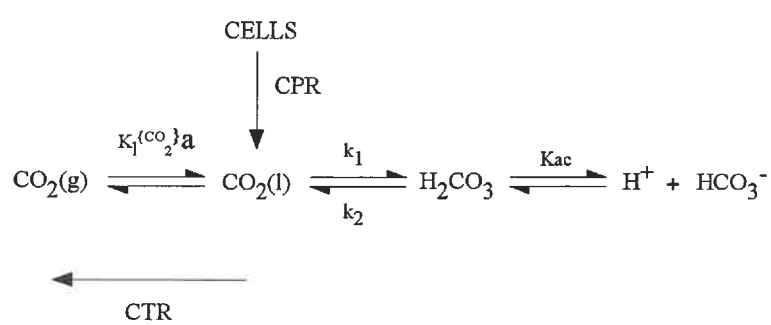

Figure a.1 Carbon dioxide pathways from biomass towards liquid and gas phases

where:

$k1$ and $k2$	=	rate constants for the indicated reactions
K_{ac}	=	carbonic acid dissociation constant
$K_L^{\{CO2\}}a$	=	fermenter volumetric CO_2 mass transfer coefficient

$(CO_2)_l$ dissociates to HCO_3^- and H^+. The equilibrium constant:

$$K = \frac{c_{HCO_3} c_{H^+}}{c_{l,CO_2}} \tag{a.7}$$

has a value of 4.71E-7 mole L^{-1} at 30°C. With the assumption of a well mixed gas phase, the amount of CO_2 accumulated as HCO_3^- can be estimated from Eq a.7:

$$c_{HCO_3^-} = \frac{K\, c_{l,CO_2}}{c_{H^+}} = \frac{K\, x_{out,CO_2}\, p_{tot}\, H_{CO_2}}{c_{H^+}} \tag{a.8}$$

Hence, the produced carbon dioxide enters the liquid phase as HCO_3^- and as $CO_2(l)$. This means that $P1$ in the space state model is the sum of both entities:

$$P1 = CO_2(l) + HCO_3^- \tag{a.9}$$

From Eq a.3, a.8, and a.9 assuming that CO_2 concentration in the liquid phase is the same as its concentration equilibrium it results:

$$P1 = x_{out,CO_2} H_{CO_2} P_{tot} \left(1 + \frac{K}{c_{H^+}} \right) \tag{a.10}$$

At low pH values (<6) or good pH control, the second term in the brackets of Eq a.10 can be neglected. In this study the contribution of the bicarbonate concentration was neglected due to good control of pH. Introduction of the correction term for bicarbonate contribution and pH variation, adds more noise to the data acquired from mass spectrometer [Tremblay 1991]. This assumption introduces no significant error into the calculations.

The carbon dioxide transfer rate (CTR) is calculated as in Eq a.11 provided the quasi steady-state conditions.

$$CTR = \left(\frac{M_{CO_2}}{V_{tp}} \right) \left(\frac{F_{out} x_{out,CO_2} - F_{in} x_{in,CO_2}}{V_l} \right) \tag{a.11}$$

Using Eq a.5 it becomes:

$$CTR = -\left(\frac{1}{V_l}\right) F_{in} \left(\frac{M_{CO_2}}{V_{tp}}\right)\left(x_{in,CO_2} - x_{out,CO_2} \frac{x_{in,inert}}{x_{out,inert}}\right) \qquad \text{(a.12)}$$

APPENDIX II

OBSERVER BASED μ ESTIMATOR FROM BIOMASS DRY WEIGHT DATA

In this appendix estimation of the specific growth rate from cell dry weight data is demonstrated. From the dynamical model (Eq 3.5) the following algorithm is proposed [Dochain 1986].

$$\frac{d\hat{X}(t)}{dt} = \hat{\mu}\, X(t) - D(t)\, X(t) + C_1\, X(t)\,[X(t) - \hat{X}(t)]$$
$$\frac{d\hat{\mu}(t)}{dt} = C_2\, X(t)\,[X(t) - \hat{X}(t)] \qquad \text{(b.1)}$$

where C_1 and C_2 are two strictly positive constants and hat (^) corresponds to the estimates of X and μ. The proof of stability and convergence of the algorithm is discussed in [Dochain 1986] . The convergence criterion is demonstrated to be:

$$C_2 < \frac{C_1^2}{4} \qquad \text{(b.2)}$$

The discrete form of this algorithm is:

$$\hat{X}(t+1) = \hat{X}(t) + \hat{\mu}(t)\,TX(t) - D(t)\,TX(t) + C_1\,TX(t)\,[X(t) - \hat{X}(t)]$$
$$\hat{\mu}(t+1) = \hat{\mu}(t) + C_2\,TX(t)\,[X(t) - \hat{X}(t)] \qquad \text{(b.3)}$$

where T is the sampling period.

The algorithm in its discrete form is stable provided that:

$$C_1 < \frac{2}{TX_{\max}} \tag{b.4}$$

where X_{max} is the maximum attainable biomass in a specific fermentation.

APPENDIX III

EXPERIMENTAL RESULTS

Table III.1 Experimental results for the fermentation Expo

Time (h)	Biomass (g/L)	Glucose (g/L)	NH_4^+ (g/L)
0	0.444	11.29	3.02
3	0.586	11.43	3.14
7	0.931	10.63	3.14
11	1.393	9.36	3.27
15	1.923	8.40	3.68
19	2.447	6.93	3.54
23	2.963	5.09	3.40
27	3.632	2.97	3.02
31	4.358	1.07	3.01
34	4.711	1.15	3.13
36	4.342	0.90	3.26
38	4.656	1.72	3.39
41	5.154	4.14	3.39
43	5.641	6.77	3.13
45	5.562	9.80	3.26
47	5.987	12.80	3.67
49	6.509	14.26	3.52
51	6.759	22.32	3.82
53	6.777	28.73	4.13
55	7.342	37.64	5.67
57	7.29	43.01	4.84
59	7.195	55.18	5.45
61	7.591	69.51	7.08
63	7.291	88.85	7.63
65	6.351	89.92	8.59
67	6.378	121.26	9.68
69	6.611	110.16	9.68

Table III.2 Experimental results for the fermentation CI[1]

Time	Biomass	Glucose	PHB	NH4	PHB%
0	0.389	14.09	0.222	2.13	548
2	0.467	10.87	0.222	2.09	448
4	0.564	11.64	0.450	2.51	445
6	0.757	13.94	0.483	2.45	324
8	0.943	8.26	0.541	2.92	310
10	1.159	10.49	0.601	2.53	218
12	1.565	10.03	0.758	2.87	183
14	1.842	0.86	0.903	1.40	76
16	2.49	0.94	1.245	1.34	54
18	3.005	0.86	1.444	1.87	62
20	3.416	0.90	1.687	1.79	52
22	4.04	0.90	1.943	2.00	50
24	5.317	0.86	2.289	1.76	33
26	6.281	0.82	2.563	1.83	29
28	6.984	0.78	2.504	1.73	25
30	7.712	8.54	3.203	3.19	41
32	9.203	12.26	3.749	2.98	32
34	10.04	12.18	3.992	3.41	34
36	11.301	17.01	4.890	3.34	30
38	13.081	22.07	5.164	3.68	28
40	13.675	23.14	6.133	4.16	30
42	14.342	18.16	6.165	4.85	34
44	15	18.23	8.005	5.23	35
46	16.994	25.83	9.798	5.49	32
48	17.4	28.00	-	5.31	31

[1]Units are the same as in Table III.1

Table III.3 Experimental results for the fermentation CII[1]

Time	biomass	NH4	glucose	PHB	PHB%
0	0.51	2.14	12.93	0	0
1	0.482	2.94	48.60	0.162	34
3.783	0.597	2.25	43.75	0.226	38
6.783	0.826	2.28	45.47	0.291	35
9.783	1.14	2.59	46.38	0.351	31
13.783	1.584	2.38	43.75	0.509	32
15.783	2.29	1.68	41.53	0.627	27
18	2.756	2.18	41.83	0.832	30
21	3.626	1.87	35.37	1.259	35
24	4.682	1.58	15.06	1.760	38
27.783	7.371	1.57	30.62	2.357	32
29.783	8.496	1.53	27.04	2.594	31
32.783	11.219	0.99	17.47	3.484	31
34.783	14.008	0.90	10.90	5.753	41
36.283	15.309	0.84	26.96	6.208	41
37.283	17.26	0.81	24.13	6.055	35
39.283	19.379	0.63	12.23	8.644	45
41.283	21.462	0.72	10.36	10.008	47
43.283	22.604	0.48	8.15	11.740	52
45.283	25.437	0.73	0.33	13.182	52
47.283	24.456	0.62	9.57	13.966	57
49.283	28.461	0.66	11.57	17.011	60
50.783	30.561	0.76	11.57	17.888	59
53.283	32.881	0.64	12.40	21.170	64
55.283	34.228	0.61	11.48	21.576	63
58.283	36.118	0.57	11.15	23.629	65

[1]Units are the same as in Table III.1

Table III.4 Experimental results for the fermentation CIII[1]

Time	Biomass	Glucose	NH4+	PHB	PHB%
0	0.259	20.90	2.44	0	0
35.35	3.131	14.86	2.10	1.336	43
38.6	3.673	13.02	2.10	1.637	45
41.6	4.303	11.34	1.94	-	-
44.6	5.014	10.98	2.02	2.390	48
46.6	5.633	10.72	1.93	2.855	51
48.6	6.465	9.84	1.95	3.342	52
51.6	7.958	9.00	1.90	3.809	48
53.6	8.733	8.80	1.97	-	-
55.6	9.498	15.42	1.94	4.765	50
57.6	10.232	14.93	1.98	5.194	51
59.6	11.262	14.87	2.02	-	-
61.6	12.275	14.26	1.99	-	-
63.85	13.067	14.79	1.99	7.006	54
65.1	13.758	15.90	1.94	-	-
67.1	15.312	15.70	1.942	7.665	50
69.1	15.694	14.43	1.87	-	-
71.1	15.79	15.012	1.89	8.065	51
73.35	18.431	15.701	2.03	9.078	49
76.1	20.017	16.303	2.16	-	-
79.1	21.137	18.12	2.16	11.095	52
82.1	22.415	21.358	2.25	12.077	54
85.1	23.693	21.747	2.52	12.368	52
87.1	22.84	22.308	2.65	7.985	35
89.1	24.283	24.188	2.65	15.590	64
92.1	24.365	24.792	2.80	15.126	62
94.85	27.049	24.473	2.95	15.606	58
98.1	26.194	23.388	2.84	-	-
101.1	27.065	22.367	2.95	15.640	58
104.1	29.01	20.61	2.84	-	-
107.1	26.88	21.964	2.90	17.202	64

[1]Units are the same as in Table III.1

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