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Control of methanol feed for Bacillus methanolicus fermentation

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CONTROL OF METHANOL FEED FOR BACILLUS METHANOLICUS FERMENTATION

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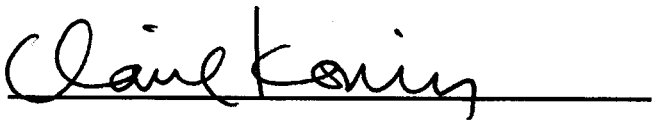

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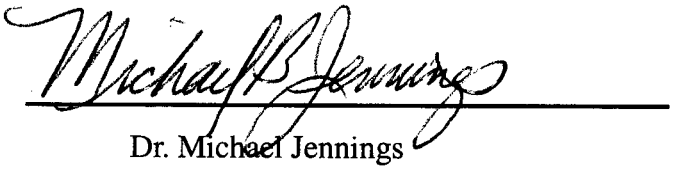

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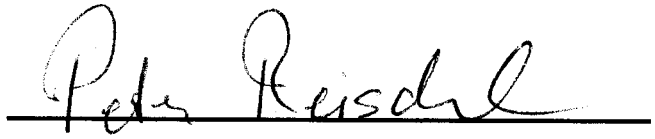

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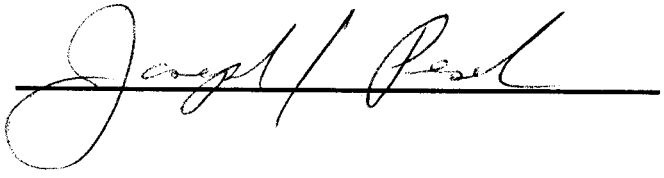

ABSTRACT

CONTROL OF METHANOL FEED FOR BACILLUS METHANOLICUS FERMENTATION

by Ching-Yun Wang

The use of thermophilic organisms as hosts to produce products of interest has been widely accepted in research and industry. *Bacillus methanolicus* is a gram positive thermotolerant organism that has been demonstrated for the production of glutamic acid and lysine. For the process control of fermentation, however, some important variables such as cell and substrate concentration are not easily to obtain on-line and this makes the process control more difficult. The use of adaptive control can overcome the disadvantage of the lack of on-line measurements and uncertainties of the process kinetics. In this paper, the dynamic model, based on material balance coupled with an asymptotic observer, is demonstrated for an adaptive control model. The design parameters in this model have been determined by experiments. This model can estimate cell and substrate concentration, specific growth rate, and enable control of the feedrate.

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NOMENCLATURE

C_L	: actual dissolved oxygen concentration
C^*	: saturated dissolved oxygen concentration
D	: dilution rate
D(0)	: D at t=0
DO	: dissolved oxygen
F	: flow rate of nutrient solution
H_{O2}	: Henry's constant
k_d	: death rate
K_{La}	: volumetric oxygen transfer coefficient
m_s	: maintenance coefficient
OD_{500}	: optical density at 500 nm
OTR	: oxygen transfer rate
OUR	: oxygen uptake rate
pH	: pH
P_{O2}	: partial pressure of oxygen
q_{O2}	: specific rate of oxygen consumption
S	: effluent substrate concentration
S(0)	: S at t=0
S_0	: feed substrate concentration
S_{in}	: feed substrate concentration for adaptive control

$\hat{S}$: on-line estimate for the substrate concentration, S

$\hat{S}(0)$: $\hat{S}$ at t=0

S^* : setpoint for S

t : time

V_R : working volume in the bioreactor

X : biomass concentration

X(0) : X at t=0

X_0 : feed biomass concentration

x_{O2} : mol fraction of oxygen in the liquid phase

$\hat{X}$: on-line estimate for the biomass concentration, X

Yx/s : yield of biomass on substrate

$Y^{AP}_{X/S}$: apparent yield

$Y^{M}_{X/S}$: maximum value of yield coefficient

Z : auxiliary variable used for adaptive control, $S + \frac{X}{Y_{X/S}}$

Z(0) : Z at t=0

μ : specific growth rate

μ_{max} : maximum growth rate

$\hat{\mu}$: on-line estimate for the specific growth rate, μ

$\hat{\mu}(0)$: $\hat{\mu}$ at t=0

γ_1, γ_2 and λ_1 : design parameters for adaptive control

CHAPTER 1.0

INTRODUCTION

1.1 What is a Bioprocess?

The most ancient bioprocess is the manufacture of beer and wine. The yeast were fed barley or grapes to result in the conversion of sugar to alcohol [1]. Without understanding the chemistry, early brewers were able to produce fine beverages. Through advanced technology, people now know that this process is accomplished by yeast and natural enzymes that can convert the carbohydrate in fruits and grains into alcohol and carbon dioxide.

Generally speaking, any single cell can be regarded as a microscopic factory. It can convert raw materials such as sugar or other simple organic molecules into products of interest via many complex reactions. From this point of view, a bioprocess is defined as a chemical process that uses microorganisms to catalyze the production of a product of interest.

Moreover, bioprocesses are widely used in the pharmaceutical industry for the production of antibodies, insulin, penicillin, vaccines, and many other drugs [2]. For the food industry, beer and wine are the best known bioproducts; however, many additives and nutrients are also produced by bioprocesses.

The best way to produce large quantities of a product of interest with a bioprocess is by using a bioreactor. A bioreactor is a tank with instrumentation that can control the cellular activity of microorganisms efficiently by controlling the environment in which the cells develop.

1.2 Bioreactor and Nutrient Feeds

There are three modes of operation of a bioreactor: batch cultivation, fed-batch cultivation, and continuous cultivation. Batch represents one limit with a single transfer of reactants into the bioreactor. Continuous represents the other extreme with constant reactant feed to and product removal from the bioreactor. In the process of fed-batch cultivation, medium components are continuously or semicontinuously fed to the reactor. Besides medium, oxygen is continuously supplied to the bioreactor, and the acid/base is also pumped to the reactor to adjust the pH. Since the cell mass is continuously increasing during the operation, the feed rate must be controlled properly by a feedback control loop or a pre-determined flow-profile. Namely, more control over the concentration of certain compounds such as the nutrients, the substrate, or the inhibitor produced by the process can be achieved. A typical bioreactor configuration is shown in Figure 1.

Cells need nutrients to grow in the bioprocess. The following materials are supplied as nutrients: carbon sources (glucose, sucrose, fructose, methanol, and ethanol),

nitrogen sources (ammonia or the ammonium salts (NH_4Cl, $(NH_4)_2SO_4$), oxygen, phosphorus, sulfur, potassium, magnesium, and other trace elements (Ca, Na, Cu).

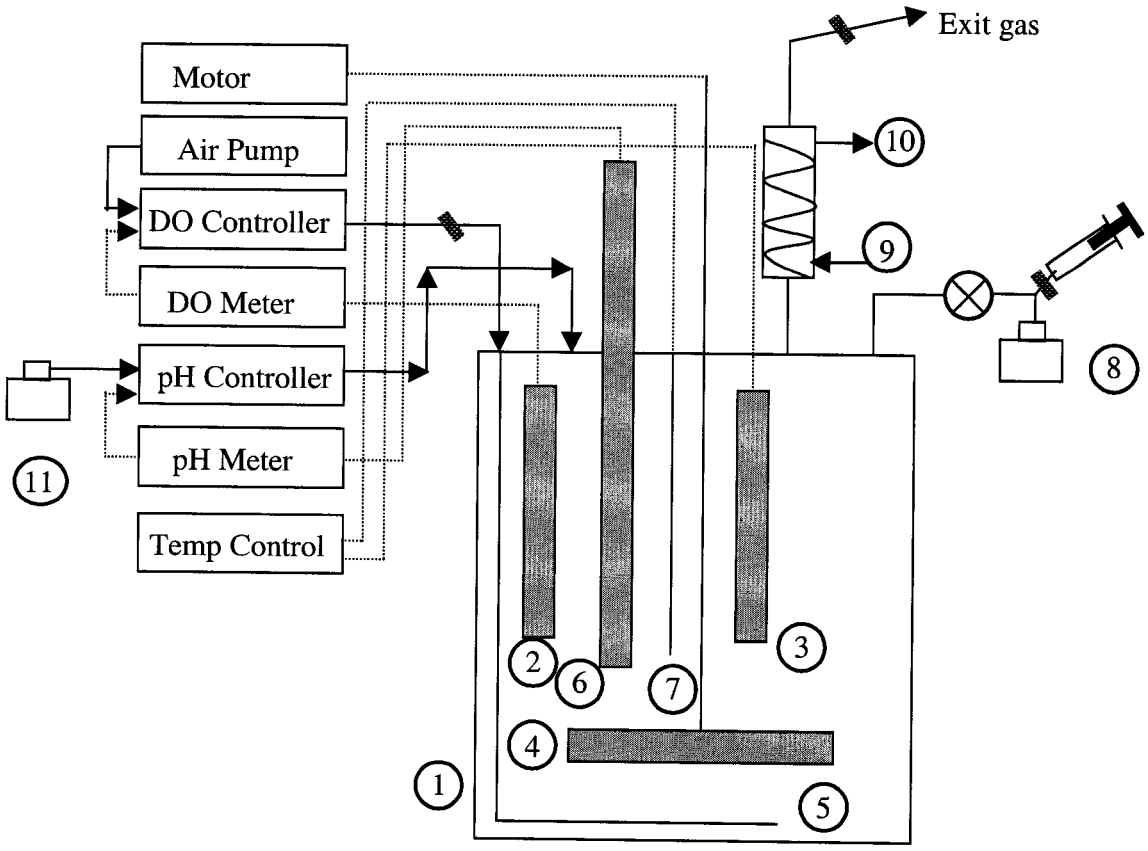

Figure 1. Typical bioreactor configuration (provided by Dr. Claire Komives)

Elements of the Apparatus	
1. Glass vessel	7. Temperature probe
2. Dissolved oxygen (DO) probe	8. Sample bottle
3. Sampling tube	9. Cooling water inlet
4. Impeller for agitation	10. Cooling water outlet
5. Sparger for air or oxygen	11. Acid/Base feed for pH adjustment
6. pH probe	

1.3 Bioprocess Control and Control System Configuration

Given reliable measurements of process variables, a process model for a laboratory-scale bioreactor can be established. Such a model could be used to predict the behavior of a bioreactor more precisely. If the model is part of a process control scheme, the following benefits can be realized: decreased overall costs, increased product yield, and improved product quality. In any control scheme, process measurements are used to analyze the state of the system, and the control model is then used to adjust critical independent variables. Some parameters can be estimated when complete process information cannot be measured directly.

There are three types of configurations used in the control systems: feedback control, feedforward control, and open loop control. In feedback control, the process is controlled by transmitting process output information back to the controller where it is compared with the setpoint value. In this configuration, controllers make decisions and take actions which affect the process. This type of control is one of the simplest and the most common structures in the process control. A typical feedback control loop is shown in Figure 2.

The independent variables of a bioreactor include the reactor feed properties (temperature, composition, concentration, and feed rate). However, the cell growth rate and product concentration are defined as dependent variables. Dissolved oxygen (DO),

pH, dissolved carbon dioxide, etc. are not exactly independent variables, but they can be controlled independently. Only few common variables are measured on-line (temperature, dO_2, dCO_2, and pH), and these variables are indirectly related to the process control. The real-time monitoring of carbon source, for example, can provide great advantages to the bioprocess control.

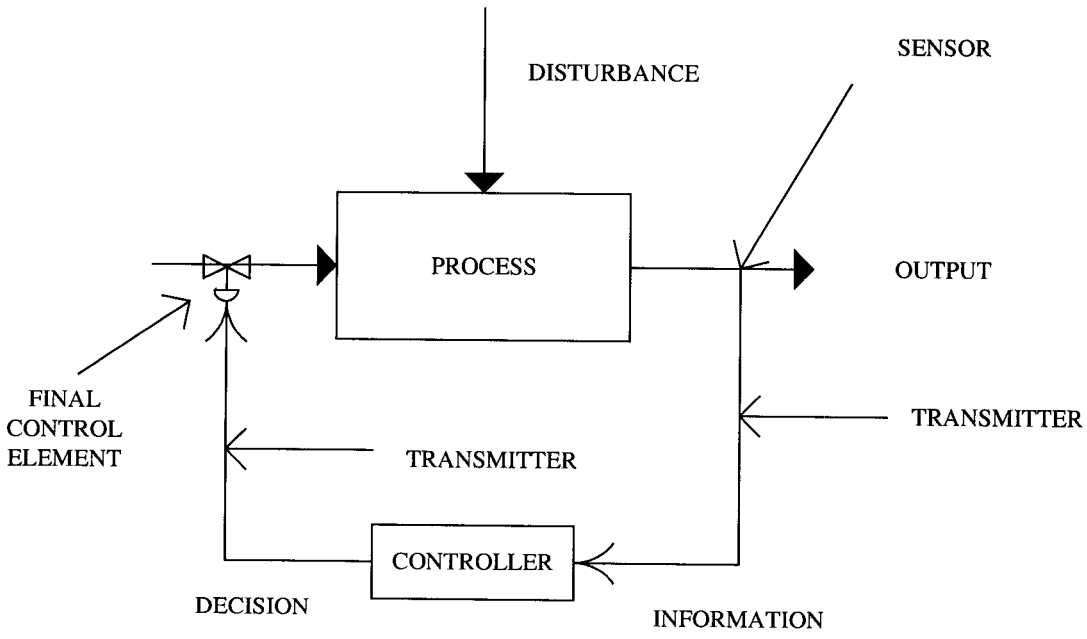

Figure 2. Feedback control loop.

Bacillus methanolicus MGA3, a gram positive bacteria, is able to use methanol as its sole carbon and energy source. It has been reported that *B. methanolicus* approaches its maximum specific growth rate μ_{max}=0.51 (hr^{-1}) at: pH 7; temperature of 50℃; continuous operation; minimal salts solution with trace metal solution; feed concentration of methanol=100 mM [3]. The reported value of μ_{max} is an acceptable growth rate for an industrial host organism. In addition, methanol is an inexpensive carbon source. Compared with E. coli and other mesophilic organisms that grow at 37℃, *B. methanolicus* also can grow above 50℃, which enables the reduction of the cooling cost. Figure 3 shows a comparison of the cooling water requirements for an organism grown at 50℃ compared to growth at 37℃ when grown on methanol. As can be seen from Figure 3, for large scale reactors, the cooling water requirement for *B. methanolicus* is around 50% less, than that for *E. coli*. The ultimate goal is to develop a mathematical model to predict the behavior of the cell with methanol feedback control in a bioreactor. After developing this model, researchers can also apply this model to other bacteria as long as they can utilize methanol as the carbon source. Model development requires an understanding about model parameters for the bioprocess, bioreactor, nutrients for cell growth, and control system configurations.

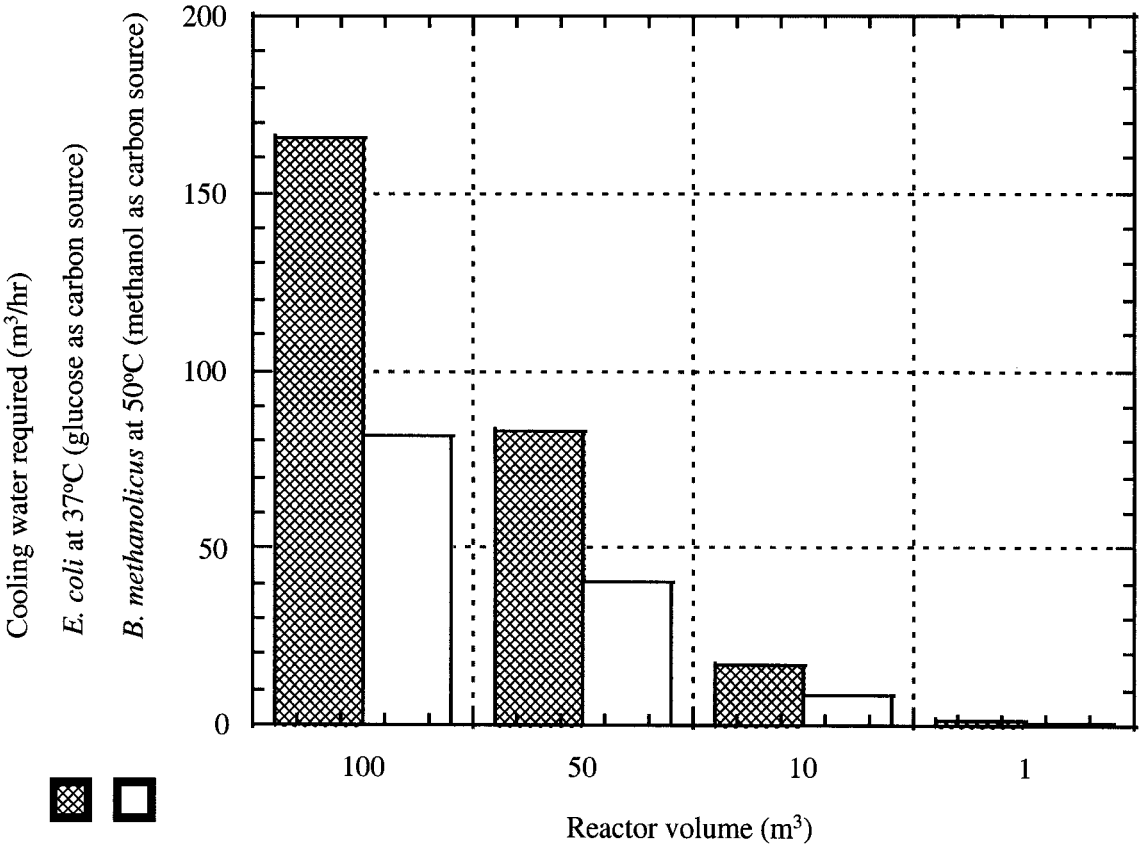

Figure 3. Cooling water requirements. The cooling water requirement was based on the difference between heat generated by bacteria and heat loss through natural convection. The inlet cooling water temperature was 20℃ and outlet cooling water temperature was three Celsius degree lower than the operating temperature.

CHAPTER 2.0

LITERATURE REVIEW

The need for the development of methods to control fermentation processes has been described in the literature [4,5]. For industrial-scale biotechnological processes, the need for monitoring systems and automatic control in order to optimize production efficiency and improve product quality in process operation is significant. Microbes are sensitive to environmental factors such as temperature, pH and nutrient concentrations. The control of temperature, pH, and dissolved oxygen is standard on most industrial fermentation systems. However, the control of the carbon and energy source is also important and has been the subject of many research programs [6-8]. This project involves the control of liquid phase methanol concentration in a fed-batch bioreactor by culture of *Bacillus methanolicus*, which can use methanol as a carbon and energy source. This organism has been demonstrated for the production of lysine (modified strain) and glutamic acid, and has been suggested for the production of single-cell protein [3]. In this project, biomass formation will be a priority for the model. This review will cover relevant methods from the literature of methanol measurement, growth models, feeding strategies, and control model development. Much of the initial work leading to this project was carried out by the research group of M. Flickinger at the University of Minnesota [3].

2.1 Methanol Concentration Measurement

Control methods are based upon a comparison between target setpoints and process measurements. Measurements of organic solvent-based carbon and energy sources for bioprocesses have been carried out in a number of ways. The method of on-line measurement of liquid methanol concentration could be accomplished either directly in the liquid with a methanol probe or indirectly by measuring the volatile methanol in the off gas. The methanol concentration in Pluschkell's research was determined by gas chromatography (Hewlett Packard, Model 5890) using a flame ionization detector and a 30 m DB-WAX column [3].

2.2 Steady-State Growth Model

Biochemical structured balances and relationships of bioenergetics have been used to develop a steady-state growth model for continuous operation [3]. Biochemical structured balances consist of several possible reactions for a process. The reactants, intermediate, and products are shown in each possible reaction. Based on material and energy balances, the stoichiometry of each reaction in a process can be completely quantified. The parameters for the reaction kinetics can then be obtained by regression of empirical data. Several groups have used this method to successively model growth with or without product formation by a variety of microorganisms [9-11]. This method was used to develop a model for a batch process to describe the production of ajmalicine,

using *Catharanthus roseus* cells. A five-parameter model was tested experimentally and proved to describe the experimental results adequately [9]. A successful model was developed to predict the dynamic behavior of a continuous culture of *Saccharomyces cerevisiae* when this process was subjected to a sudden increase in the dilution rate [10].

Often times, there is a trade-off between the yield and the productivity in bioprocess. F. Jadot et al. [12] developed a method to model the yield-productivity conflict in a bioprocess. This research used two biological reactions to simulate a bioprocess; a reaction for the conversion of substrate to biomass, and a second reaction for the conversion of substrate to extracellular product. Two performance criteria were used to optimize the process: the productivity Jp which was the production per unit of time and the yield Y_p which was the production per unit of substrate fed to the reactor.

For analysis and design of a bioprocess, one must consider the mechanism of biological reactions. The most important issue for biological reactions is the mass transfer of material to and from the surface of the microbial cells. Mass transfer was incorporated into a number of models for growth kinetics, substrate uptake kinetics, and inhibition kinetics [4]. A growth model for *B. methanolicus* has been developed by Pluschkell [3] that is appropriate for the control model development in this paper. Her model employed simultaneous reactions that represent the carbon and energy flow for the production of biomass and glutamic acid. The growth model can be modified to include only biomass production and any significant metabolic byproducts.

2.3 Feeding Strategy

It is important to know the effect of methanol on a cell because a high concentration of substrate (methanol) may cause inhibition or toxicity to the cell. To avoid this situation, it is important to understand how the methanol concentration affects the growth of *B. methanolicus*.

Several feeding strategies have been tested for microbial reactions. Jovan D. Boskovic summarized the main drawbacks for different common feeding strategies such as the exponential feeding strategy (EFS), linear feeding strategy (LFS), and the feedback exponential feeding strategy (FEFS) [13]. Results of this research showed that the modified linear feeding strategy can provide a more accurate simulation than other feeding strategies. One of the disadvantages of this design was that both cell and substrate concentrations were needed to implement the control loop.

The methanol concentration effect was investigated under batch conditions [3]. This research found the biomass concentration in shake flasks was a function of the initial methanol concentration within a limited range of initial methanol concentration (60 mM to 220 mM). The dry biomass concentration increased steadily from 0.9 g/l at 60 mM methanol in the initial charge to 2.6 g/l at 220 mM methanol in the initial charge. The growth rate measured for *B. methanolicus* MGA3 in these experiments changed from 0.51 hr^{-1} at 60 mM methanol in the initial charge to 0.45 hr^{-1} at 220 mM methanol in the

initial charge. Based on these results, it appears that the initial methanol concentration did not play an important role in the growth rate. It can also be seen that the initial methanol concentration in this range (60 mM to 220 mM) did not cause inhibition or toxicity to the growth of *B. methanolicus* MGA3.

The work of Plushkell [3] as described above, did not define an optimal initial methanol charge concentration. The optimal concentration of methanol may change over the course of the run and this issue has been approached in a number of ways. For the culture of the *osmophilic* yeast, Fan [14] found that high glucose concentration inhibited the cell growth but low concentration reduced the productivity. Dong-Ming Xie et al. [15] reported the glucose feed control strategies for a fed-batch culture of osmophilic yeast *Candida krusei* and they tried to divide the entire fed-batch process into multi-subinterval because in that way they can feed the glucose in pulse form and keep the temperature at a constant value in each subinterval. Compared with other empirical strategies, their method provides a basis to improve the productivity significantly.

2.4 Control Model Development

B. Ogunnaike and W. H. Ray [16] have presented the general concepts for process modeling and control in their book. After setting up all variables (output, control, disturbance and state variables), and differential equations (the growth model), data can be converted to mathematical expressions (Laplace transform or Fourier transform), and then these mathematical expressions can be used to control the system. Options for the approach to process control include: linear and nonlinear control; adaptive and non-adaptive control, and the methods to estimate parameters in the control model.

For a typical bioprocess, the development of the process control model is a complex exercise and always needs considerable mathematical modeling. In addition, the dynamics of bacteria are often poorly understood and this makes the control of bioprocesses more difficult. G. Bastin and D. Dochain [17] demonstrated the application of mathematical modeling techniques and dynamical systems analysis in the solution of engineering problems in bioreactors. In order to overcome the disadvantage of a lack of on-line measurements and uncertainties in the kinetic model, an adaptive control model was developed by these researchers, based on the dynamical model (material balance) coupled with an asymptotic observer. Their adaptive control model can estimate the parameters to model biomass concentration, specific growth rate, substrate concentration, and dilution rate by measuring either biomass concentration or substrate concentration. F. Jadot et al. [12] and J. D. Boskovic [13] also employed this

adaptive control model in their research. R. Thatipamala et al. [18] developed a control algorithm to predict the unmeasured state variables such as the specific growth rate, biomass yield, and product yield. In their research, the glucose concentration was measured every 0.5 min and was used to implement the feed rate. In addition, the biomass yield on glucose was continuously modified based on known growth kinetic models during the experiment. An asymptotic observer was also employed to provide the on-line estimation of biomass concentration, glucose concentration, and product concentration, and results were within ±5% error of off-line measurements.

CHAPTER 3.0

RESEARCH HYPOTHESIS AND OBJECTIVES

3.1 Research Hypothesis

B. methanolicus consumes methanol as its sole carbon and energy source. The hypothesis for this research is that concentration of methanol in the broth would affect the growth behavior of *B. methanolicus* and the methanol concentration could be manipulated to optimize the bacteria for maximum growth and biomass production.

3.2 Research Objective

Monitor the consumption of methanol by *B. methanolicus* and use this information to control the liquid phase methanol concentration. Define the optimal methanol concentration for achieving maximum growth and biomass production.

CHAPTER 4.0

MATERIAL AND METHODS

4.1 Microorganism

Bacillus methanolicus, a thermophilic methyotrophic bacteria, was used in this study, and the growth medium has been reported by Pluschkell [3] and can be found in Tables 1 through 3. According to the American Type Culture Collection (ATCC) protocol, trypticase soy broth was used to grow the freeze-dried material [19]. For the starter culture, *B. methanolicus* was inoculated in a 125 ml baffled shake flask and this shake flask was put in an incubator with a shaking speed of 300 rpm for 16 hours. The operating temperature and pressure were 50℃ and 1 atm, respectively.

Table 1. Trace metal solution (TM, 1000X).

Compound	Concentration
$FeCl_2*4H_2O$	20 mM
$ZnCl_2$	1 mM
$MnCl_2*4H_20$	50 mM
$CaCl_2*2H_2O$	50 mM
$CuCl_2*2H_2O$	160 μM
$CoCl_2*6H_2O$	170 μM
$Na_2MoO_4*2H_2O$	200 μM
H_3BO_3	490 μM

Table 2. Minimal salts solution (MS).

Compound	Composition
$K_2HPO_4*3H_2O$	6.47 g/l
$NaH_2PO_4*H_2O$	1.89 g/l
$(NH_4)_2SO_4$	3.6 g/l

Table 3. Medium for growth of *B. methanolicus* in batch culture.

Medium component	Amount added
Minimal salts solution (MS)	1000 ml
Yeast extract	0.5 g
Trace metal solution (TM, 1000X)	1.0 ml
$MgSO_4*7H_2O$	0.25 g
Biotin	20 µg
Vitamin B_{12}	1 µg
Methanol	Varies

The effect of methanol concentration on the growth of *B. methanolicus* was determined as follows: six shake flasks with different initial methanol concentrations (0.8, 3.2, 4, 8, 12, and 16 g/l) were used to examine the methanol concentration effects on biomass concentration and growth rate. A UV/Visible spectrophotometer was used to measure the optical density of the culture broth every hour and sampling procedures are described in Section 4.4. The initial growth rate can be determined from the optical density data using the relationship shown in Equation 1. The optical density data were also used to measure the biomass concentration using the function shown in Equation 2. Results from the analyses were used to determine the initial methanol concentration for maximum biomass formation. This optimal growth initial methanol concentration was applied to subsequent experiments.

$$\mu \equiv \frac{1}{X}\frac{dX}{dt} \qquad \text{Equation 1}$$

where X is biomass concentration (g/l), t is time (hr), and μ is the specific growth rate (hr^{-1}).

$$\text{Dry biomass} = m^{*}OD_{500} \qquad \text{Equation 2}$$

where m is the slope of dry biomass (y-axis) versus optical density (x-axis).

4.2 Bioreactor

The bioreactor was configured according to Figure 1, as shown in Section 1.2, and the operating conditions are shown in Table 4. The composition of the growth medium used as feed in fed-batch and continuous operations is shown in Table 5. The bioreactor system included Biocontroller (Applikon, Model ADI 1030), Bioconsole (Applikon, Model 1035), and a 3-liter bioreactor. Reaction operating conditions were monitored using a pH probe, a dissolved oxygen probe, and a thermocouple to measure the temperature of the medium. Acid or base were pumped into the reactor to control pH. The reaction temperature was controlled by employing a heating jacket to maintain setpoint values. Bacteria growth rate was independent of dissolved oxygen (DO) concentration for an aerobic culture for values above a critical oxygen concentration. The DO value was maintained above 20% which exceeded the critical oxygen concentration for the culture. The methanol feed rate was the only remaining independent control variable in this study and the liquid methanol concentration of the medium was the measured output variable.

Table 4. Operating conditions for the bioreactor.

Process variables	Setpoints
pH	6.6
Temperature	50℃
Dissolved Oxygen (DO)	>20%

Table 5. Medium for growth of *B. methanolicus* in fed-batch and continuous culture.

Medium component	Amount added
Minimal salts solution (MS)	1000 ml
Trace metal solution (TM, 1000X)	1.0 ml
$MgSO_4$	0.25 g
Biotin	20 μg
Vitamin B_{12}	1 μg
Methanol	3.2 g

An autoclave was used to sterilize the bioreactor and growth medium and standard sterilization procedures were applied. According to the procedures described in the Applikon manual, the standard calibration procedures were employed to calibrate the feed pumps and measuring probes.

4.3 Methanol Concentration Measurement

The biochemistry analyzer (YSI, Model 2700 SELECT) was used to measure the liquid methanol concentration off-line and 25 μl of sample was needed for each measurement. The YSI analyzer was calibrated with standard solutions before sample analyses, using procedures specified by the manufacturer.

4.4 Biomass Measurement

A UV/Visible spectrophotometer (Hewlett Packard, Model 8452A, 1 cm path length) was used to measure the optical density (OD_{500}) of culture broth samples. Biomass concentration was correlated with absorbance measured at a wavelength of 500 nm. A micropipette was used to transfer 1 ml samples from shake flasks or sample bottles to the 1.5 ml cuvettes used for the UV/Visible spectrophotometer. To ensure measurement accuracy, the optical density value was kept below 0.5 by diluting the sample with distilled water.

The dry biomass concentration of a culture suspension was measured by centrifugation of 25 ml of samples for 10 minutes followed by supernatant discard. The resulting cell pellets were resuspended in 12 ml minimal salts solution (MS) and centrifuged for another 10 minutes. The resulting cell pellets were resuspended again in 12 ml MS and then dried at 80°C in aluminum cups to a constant mass. The quantitative relationship between the measured optical densities of the culture broth and the dry biomass concentrations was correlated using Equation 2.

4.5 Oxygen Transfer Rate and Oxygen Uptake Rate

4.5.1 Oxygen Solubility

The oxygen solubility for low oxygen concentrations in liquid can be calculated by Henry's law:

$$P_{O2} = H_{O2} \times x_{O2} \qquad \text{Equation 3}$$

where P_{O2} is the partial pressure of oxygen in the gas phase, H_{O2} is the Henry's constant which depends on the temperature of the system, and x_{O2} is the mol fraction of oxygen in the liquid phase. The Henry's constant at 50℃ for oxygen solubility in water from Perry's chemical engineering handbook (Table 2-140) is 5.88×10^{-4} [20]. The oxygen solubility under ambient air at one atmosphere can be calculated by Henry's law at standard operating condition as 0.1981 mmol/l or 6.34 mg/l. From this calculation, the saturated dissolved oxygen concentration in the reactor, C^*, is set to be 6.34 mg/l.

4.5.2 Oxygen Transfer Rate

Equations 4 through 12 are standard biochemical engineering balances and can be found in reference [2]. Oxygen transfer rate (OTR) is the amount of oxygen transferred from the bubbles into the liquid and can be expressed as:

$$OTR = K_{La}(C^* - C_L) \qquad \text{Equation 4}$$

where OTR is the oxygen transfer rate (mg/l-min), K_{La} is the volumetric oxygen transfer coefficient (hr^{-1}), C^* is saturated dissolved oxygen concentration (mg/l), and C_L is the actual dissolved oxygen concentration (mg/l). The value of K_{La} does not vary for constant operating conditions and can be determined by plotting C_L versus time.

4.5.3 Oxygen Uptake Rate and the Specific Rate of Oxygen Consumption

The specific rate of oxygen consumption (q_{O2}) and oxygen uptake rate (OUR) can be estimated via the dynamic method. The dissolved oxygen values in a 3-liter bioreactor were recorded every minute. When the bioreactor reached a steady-state, the air supply was shut off for 4 minutes and then turned back on to determine q_{O2}. The estimation of OUR and q_{O2} are based on the following equations:

$$\frac{dC_L}{dt} = OTR - OUR = K_{La}(C^* - C_L) - q_{O2}X \qquad \text{Equation 5}$$

where OUR is the oxygen uptake rate (mg/l-min) by the bacteria and q_{O2} is the specific rate of oxygen consumption (mg O_2/ g cells-min).

When the air was turned off (OTR=0), the DO concentration decreased because the DO was consumed by the cells in the bioreactor. The consumption rate can be expressed as:

$$\frac{dC_L}{dt} = -q_{O2}X = OUR \qquad \text{Equation 6}$$

The slope for the plot of DO versus time is OUR or $-q_{O2}X$. OUR and q_{O2} can be determined by measuring X and recording the DO concentration with time.

4.6 The Biomass Yield on Methanol (Yx/s) under Steady-State Operation

Chemostat operation:

The material balance on the biomass concentration in the bioreactor is:

$$FX_0 - FX + V_R\mu\ X - V_R k_d X = V_R \frac{dX}{dt} \qquad \text{Equation 7}$$

where F is the flow rate of nutrient solution (hr^{-1}), V_R is the working volume in the bioreactor (liter), X_0 and X are feed and effluent biomass concentration (g/l), and μ and k_d are growth and death rate constants (hr^{-1}).

Equation 7 can be resolved to yield:

$$\frac{dX}{dt} = DX_0 + (\mu - k_d - D)X \qquad \text{Equation 8}$$

where D is dilution rate and $D=F/V_R$. Under steady-state conditions ($dX/dt=0$ and $X_0=0$), the dilution rate can be expressed as:

$$D = \mu - k_d \qquad \text{Equation 9}$$

In a chemostat, the cell growth rate can be manipulated by changing the dilution rate.

The material balance on the substrate concentration can be expressed as:

$$FS_0 - FS - V_R \mu X \frac{1}{Y^M{}_{X/S}} = V_R \frac{dS}{dt} \qquad \text{Equation 10}$$

where S_0 and S are feed and effluent substrate concentration (g/l), μ is the specific growth rate (hr^{-1}), and $Y^M{}_{X/S}$ is the maximum value of yield coefficient (g cells/g S). Under steady-state conditions ($dS/dt=0$), Equation 10 can be rearranged as:

$$D(S_0 - S) = \frac{\mu X}{Y^M{}_{X/S}} \qquad \text{Equation 11}$$

Substituting Equation 9 into Equation 11 and rearranging yields for steady-state operation:

$$D\left(\frac{S_0 - S}{X}\right) - \frac{D + k_d}{Y^M{}_{X/S}} = 0$$

$$D\left(\frac{1}{Y^{AP}{}_{X/S}}\right) - \frac{D}{Y^M{}_{X/S}} - \frac{k_d}{Y^M{}_{X/S}} = 0$$

$$\frac{1}{Y^M{}_{X/S}} + \frac{k_d}{Y^M{}_{X/S}}\frac{1}{D} = \frac{1}{Y^{AP}{}_{X/S}}$$

$$\frac{1}{Y^{AP}{}_{X/S}} = \frac{1}{Y^M{}_{X/S}} + \frac{m_s}{D} \qquad \text{Equation 12}$$

where $m_s = \frac{k_d}{Y^M{}_{X/S}}$, m_s is the maintenance coefficient based on substrate S, and $Y^{AP}{}_{X/S}$ is the apparent yield, which varies with growth condition.

4.7 Adaptive Control Model

An adaptive control model was developed by Bastin and Dochain [17] which was used for this work. Adaptive control has the capacity to modify control model parameters based on changes in measured inputs. The model uses an asymptotic observer which is used to reconstruct the missing information such as the reaction rate and some state variables.

A simple microbial growth process can be expressed as:

$$S \rightarrow X \qquad \text{Equation 13}$$

Based on the material balance, the general dynamic model for the growth process can be written as:

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}}\mu X - DS + DS_{in} \qquad \text{Equation 14}$$

$$\frac{dX}{dt} = \mu X - DX \qquad \text{Equation 15}$$

An asymptotic observer is used to provide the on-line estimate of the biomass concentration [17]. First, an auxiliary variable Z is defined as:

$$Z = S + \frac{X}{Y_{X/S}} \qquad \text{Equation 16}$$

From the mass balance Equations 13 and Equation 14, the derivative of Z with respect to t can be approximated as:

$$\frac{dZ}{dt} = DS_{in} - DZ \qquad \text{Equation 17}$$

The on-line estimate $\hat{X}$ for the biomass concentration, X, is given as:

$$\hat{X} = Y_{X/S}(Z - S) \qquad \text{Equation 18}$$

The on-line estimate $\hat{\mu}$ for the specific growth rate, μ, and $\hat{S}$ for the effluent substrate concentration, S, are given as follows:

$$\frac{d\hat{S}}{dt} = -\hat{\mu}(Z - S) + DS_{in} - DS + \gamma_1(S - \hat{S}) \qquad \text{Equation 19}$$

$$\frac{d\hat{\mu}}{dt} = -\gamma_2(S - \hat{S}) \qquad \text{Equation 20}$$

where γ_1 and γ_2 are design parameters and have to be correlated to obtain the best on-line estimates.

With this model, only one process variable, S, needs to be measured. The biomass concentration, X, and the specific growth rate, μ, can be estimated on-line using this model. Three design parameters (γ_1, γ_2, and λ_1) need to be correlated by experiments. The performance of this adaptive model depends on three design

parameters and the initial conditions. The optimal value of γ_2 can be expressed in terms of γ_1 [17]:

$$\gamma_2 = \frac{\gamma_1^{\,2}}{4(Z-S)} \qquad \text{Equation 21}$$

The adaptive controller function for the dilution rate is then expressed as follows:

$$D = \frac{\lambda_1(S^* - S) - \hat{\mu}(Z-S)}{S_{in} - S} \qquad \text{Equation 22}$$

where S^* is the setpoint for S and λ_1 is the design parameter.

CHAPTER 5.0

RESULTS AND DISCUSSION

5.1 Methanol Evaporation in Shake Flasks

Six baffled shake flasks with different initial methanol concentrations were used to determine the methanol evaporation rate. The liquid methanol concentration was measured by the YSI analyzer as described in Section 4.3. The relationship between residual methanol and time is shown graphically in Figure 4. The initial methanol evaporation rate was calculated as the slope of the residual methanol concentration versus time line for different initial methanol concentrations. Methanol evaporation rates as a function of initial methanol concentration are summarized in Table 6. A linear correlation between methanol evaporation rates and initial methanol concentrations is graphically shown in Figure 5. The equation for this linear relationship is evaluated as:

Initial methanol evaporation rate (g/l/hr)=(0.01121±0.00024)×methanol concentration (g/l).

This relationship is used to estimate the methanol evaporation loss for the growth of *B. methanolicus* in all shake flask experiments.

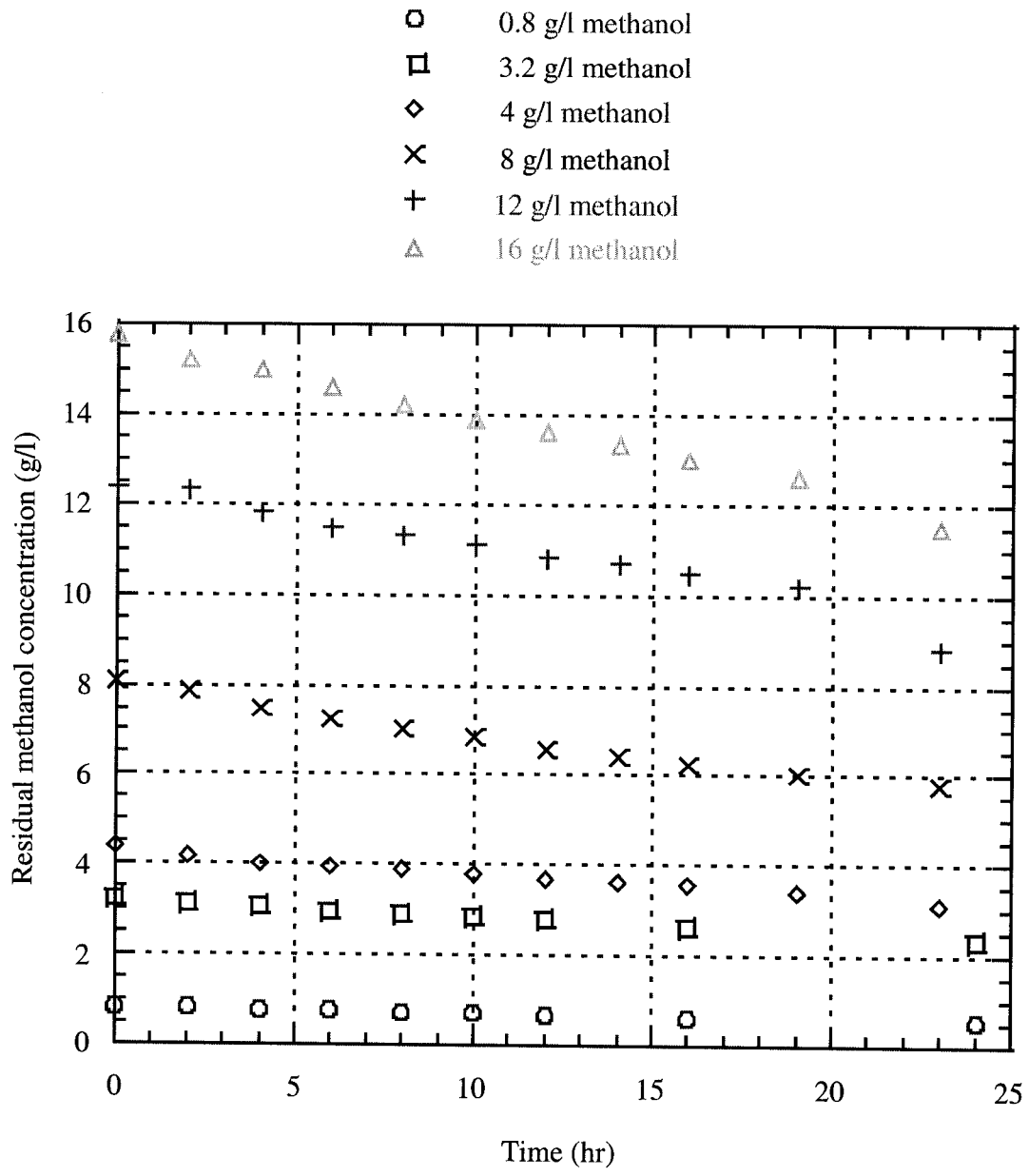

Figure 4. Methanol evaporation in shake flasks under different initial methanol concentrations.

Table 6. Initial methanol evaporation rate under different initial methanol concentrations.

Initial methanol concentration (g/l)	Initial methanol evaporation rate (g/l/hr)
0.85	-0.0134±0.0008
3.23	-0.0367±0.0008
4.43	-0.0494±0.0027
8.11	-0.1014±0.0055
12.4	-0.1374±0.0102
15.8	-0.1728±0.0054

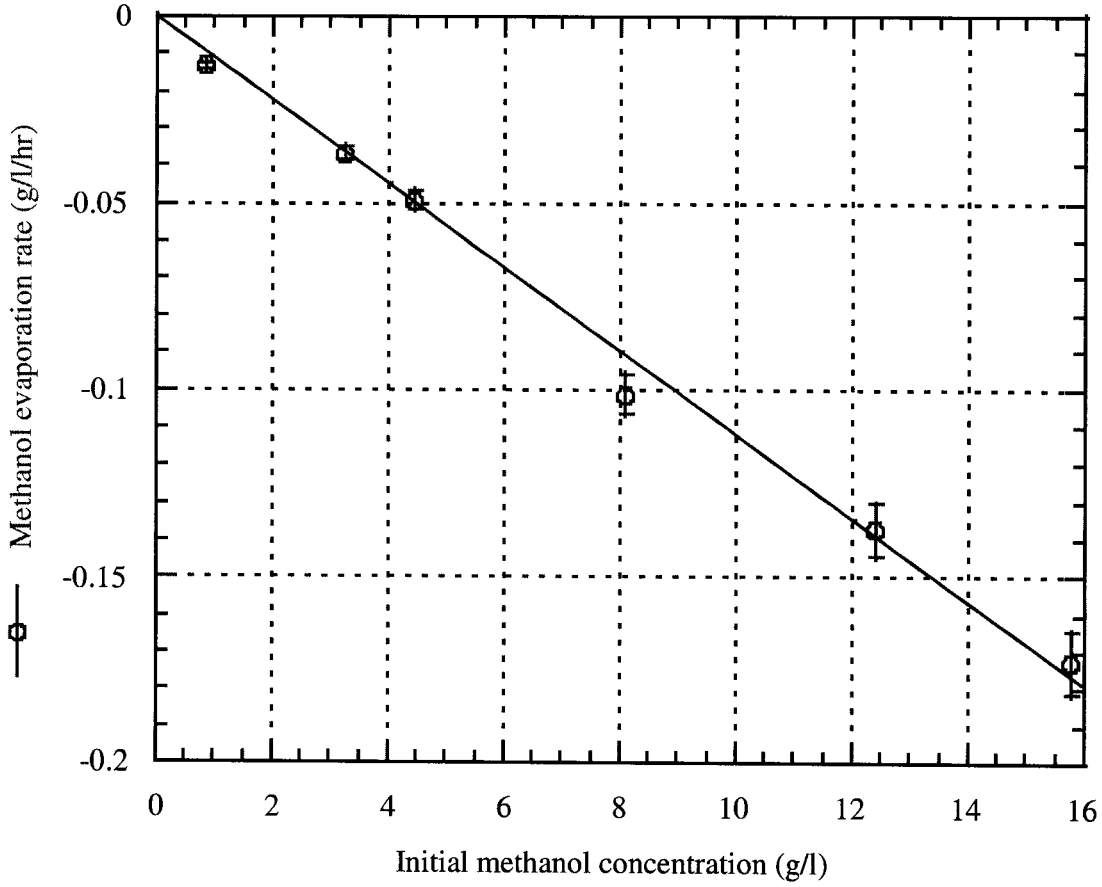

Figure 5. Methanol evaporation rate in shake flasks under different initial methanol concentrations.

5.2 The Quantitative Relationship between the Optical Density and the Dry Biomass Concentration

The method to determine the quantitative relationship between the optical density and the dry biomass concentration was described in Section 4.4. The results of dry biomass and OD_{500} measurements are shown graphically in Figure 6. The relationship between dry biomass concentration and OD_{500} can be determined by linear regression to be:

$$\text{Dry biomass} = (0.1434 \pm 0.0045) \times OD_{500} \quad (g/l) \qquad \text{Equation 23}$$

This quantitative relationship is used to measure dry biomass concentration in this research. Pluschkell [3] showed a similar quantitative relationship between the optical density and the dry biomass concentration: Dry biomass=$(0.176 \pm 0.004) \times OD_{500}$.

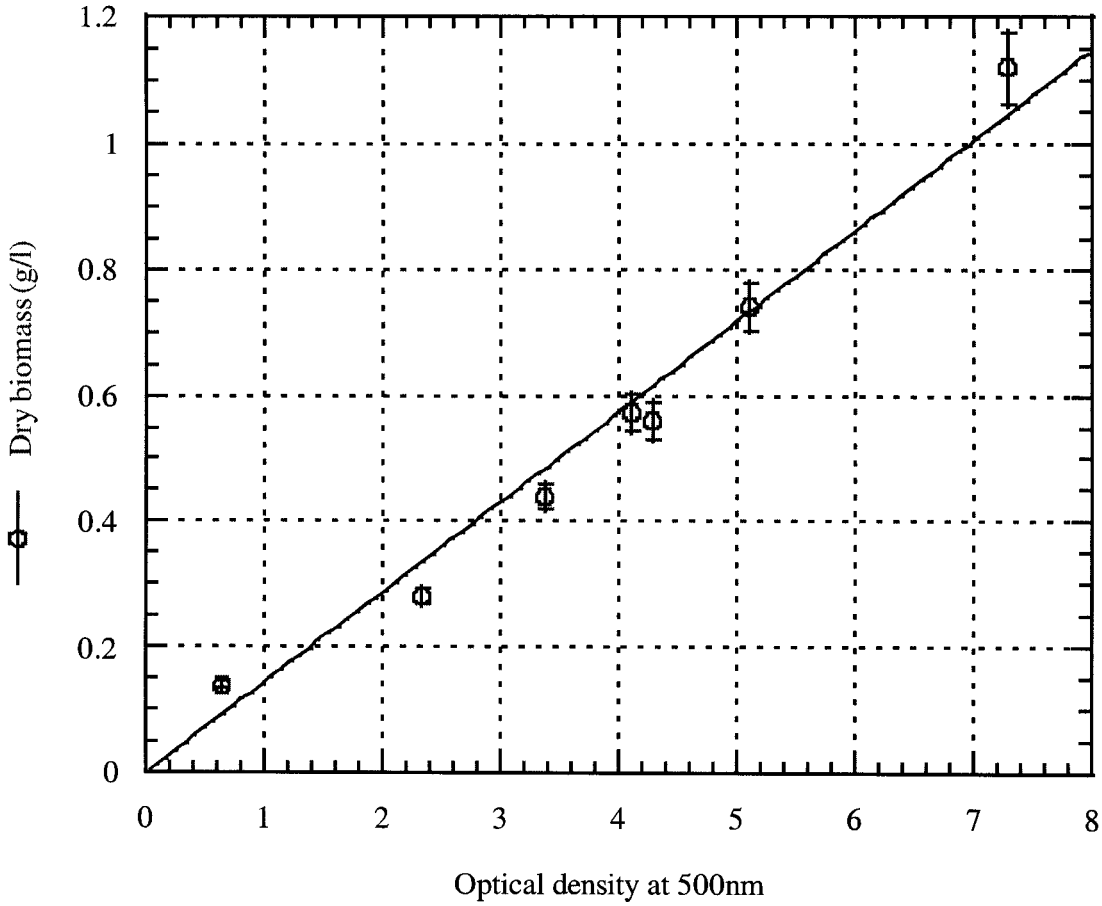

Figure 6. The estimation of dry biomass concentration of *B. methanolicus* from optical density measurements.

5.3 *B. Methanolicus* Growth in Shake Flasks

The initial methanol concentration effect on initial specific growth rate, biomass formation, and the yield of biomass on methanol, Yx/s (g cells/g methanol), has been based on experiments in 125 ml baffled shake flasks with 50 ml growth medium. The growth medium and operating conditions are provided in Section 4.1. The starter culture was inoculated under the same operating condition for 16 hours. Biomass accumulation and the residual methanol concentration were measured at different times during each run. These results are shown graphically in Figure 7 and Figure 8 for biomass accumulation and residual methanol concentration, respectively. Initial specific growth rates were determined using the initial twelve hours of incubation evaluated with Equation 1. Initial specific growth rates as a function of different initial methanol concentrations are summarized in Table 7 where the trend is that the initial specific growth rate increased with higher initial methanol concentration. Table 8 shows the biomass yield, Yx/s, as a function of different initial methanol concentrations. These data indicate a decrease in biomass yield with an increase in initial methanol concentration.

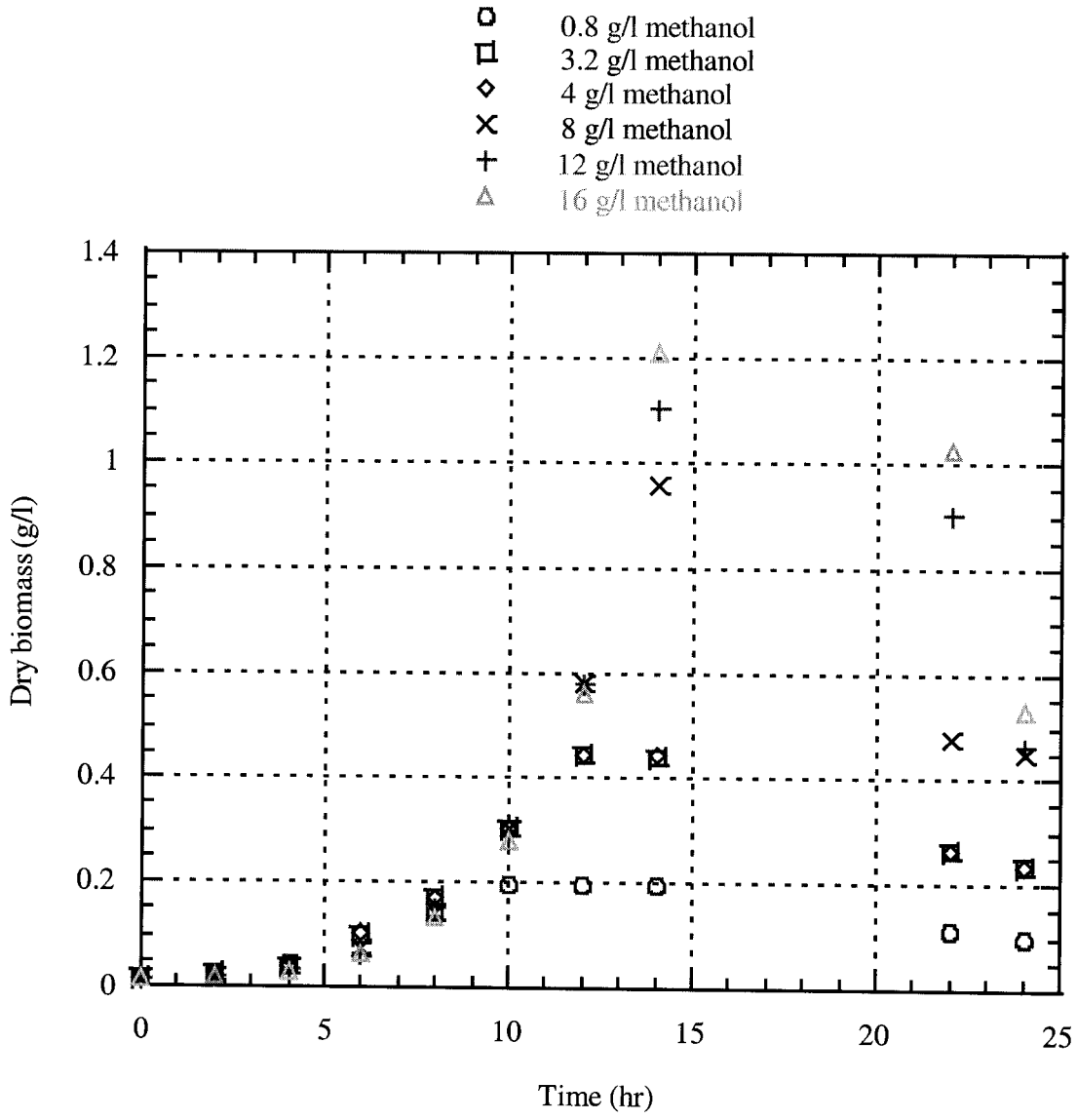

Figure 7. Growth of *B. methanolicus* in shake flasks under different initial methanol concentrations, dry biomass versus time.

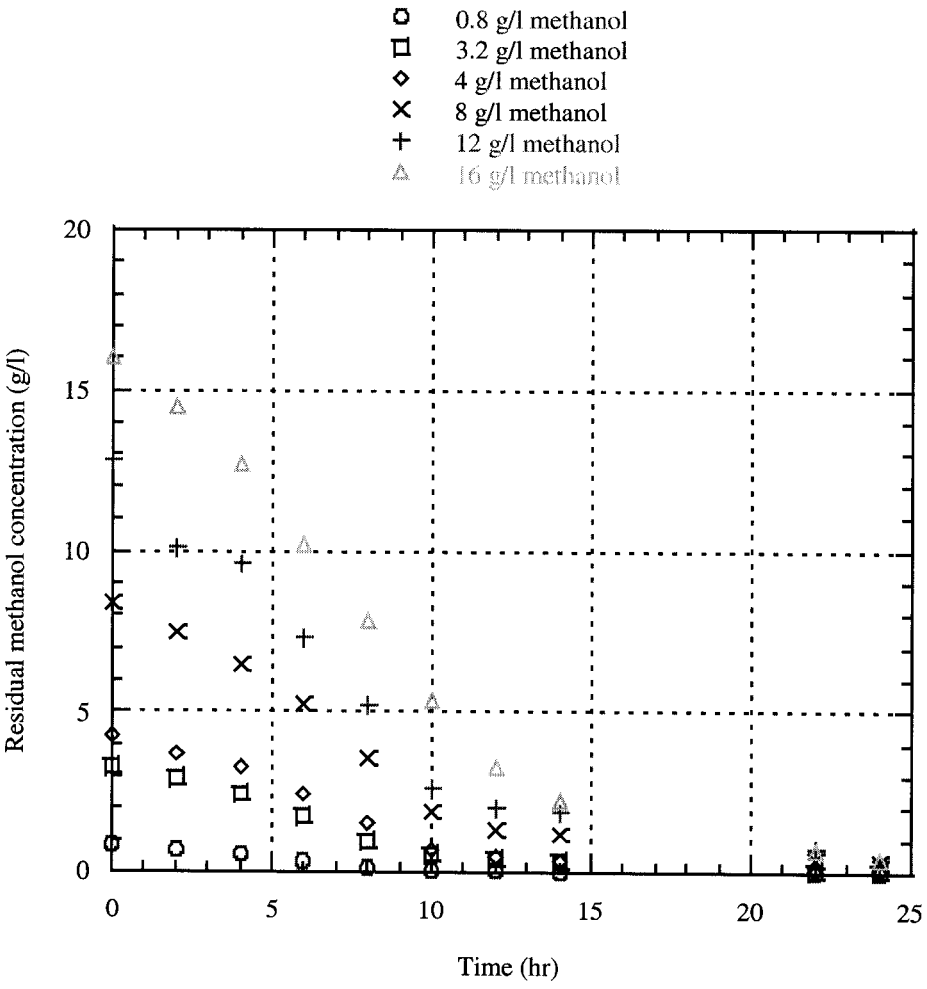

Figure 8. Growth of *B. methanolicus* in shake flasks under different initial methanol concentrations, residual methanol versus time.

Table 7. Dependence of the initial specific growth rate, μ_{max}, of *B. methanolicus* on the initial methanol concentration in shake flasks.

Initial methanol concentration (g/l)	Initial specific growth rate (hr^{-1})
0.8	0.228±0.023
3.2	0.256±0.014
4	0.251±0.016
8	0.295±0.013
12	0.391±0.036
16	0.435±0.033

Table 8. Yield of biomass on methanol, Yx/s, under different initial methanol concentrations.

Initial methanol concentration (g/l)	Yield of biomass on methanol, Yx/s (g cells/g methanol)
0.8	0.246
3.2	0.164
4	0.128
8	0.147
12	0.111
16	0.098

5.4 Oxygen Transfer in a 3-liter Bioreactor

The method used to determine OTR was described in Section 4.5.2. A 3-liter bioreactor with one liter growth medium was used to determine OTR and the operating conditions are shown in Table 4. The bioreactor was saturated with oxygen for two hours before this experiment. As shown in Figure 8, the air was turned off between t=21 to 60 minutes and pure nitrogen (1l/min) was used to degas the oxygen in the liquid phase. At t=61 minutes, the air was turned on and the nitrogen was turned off. The relationship between DO and time was recorded. Coupled with Equation 4 and Figure 9, the value of K_{La} under this operating condition was determined to be 0.18 min^{-1}. This K_{La} was used to estimate the OUR in this research.

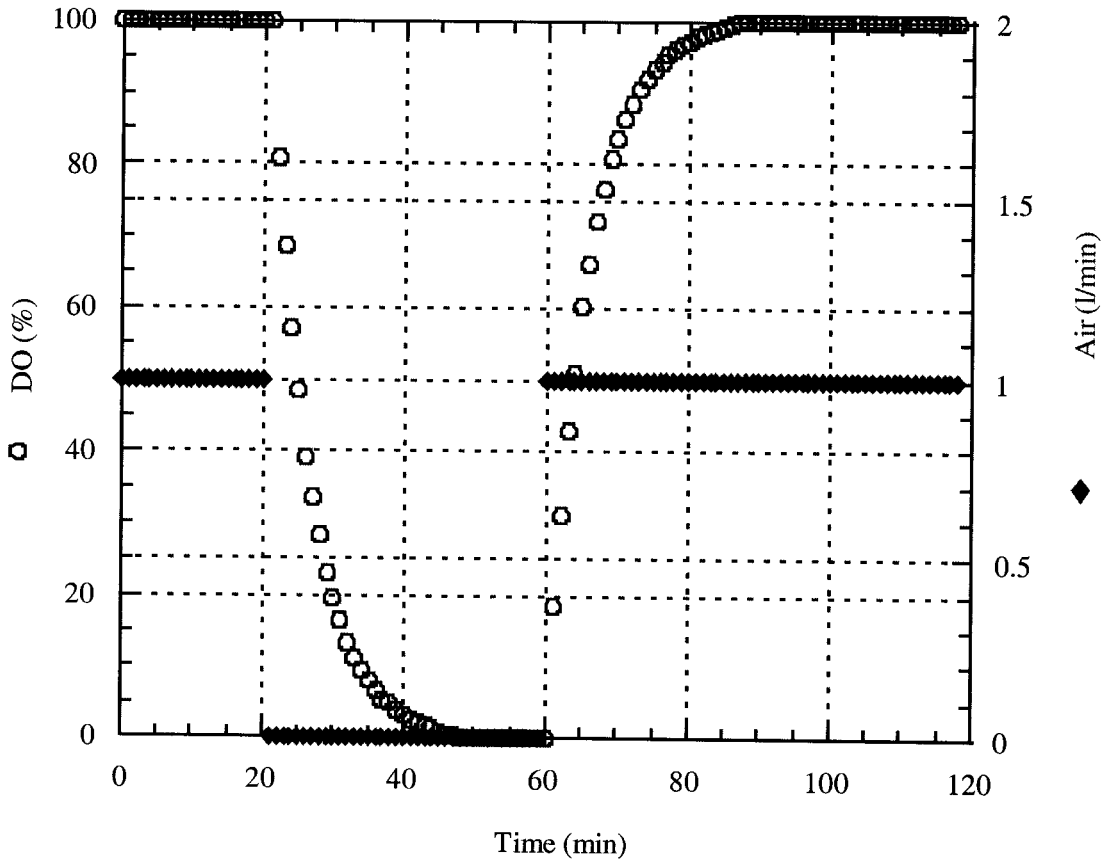

Figure 9. Oxygen transfer in a 3-liter bioreactor with one liter growth medium. The stirrer rate was fixed at 600 rpm.

5.5 *B. Methanolicus* Growth in a 3-liter Bioreactor

The inoculum which was used in the bioreactor was prepared in 125 ml shake flasks with 50 ml growth medium and incubated with shaking for 12 hours at 50℃. One liter growth medium with 3.2 g/l methanol was used to grow *B. methanolicus* in a 3-liter bioreactor. Growth medium and operating conditions are shown in Tables 1 through 4. Temperature, pH, and DO concentration were measured by different probes and were controlled by Applikon Biocontroller ADI-1030. Optical density and methanol concentration were measured during the run. Before inoculation, the bioreactor was operated under a pre-run mode for at least four hours to make sure it reached a stable condition.

5.5.1 Batch Operation

Figure 10 shows the growth of *B. methanolicus* under batch operation. The optical density and DO did not change significantly between t=0 to t=12 hours which indicated a lag phase. The exponential growth phase is observed between t=12 to t=18 hours based on the OD measurements. In addition, both methanol concentration and DO decreased dramatically within this period indicating that *B. methanolicus* entered the exponential growth phase. A sudden increase in DO between t=18 to t=20 hours is observed where a short stationary phase may have take place. After t=20 hours, the OD decreased with time which indicated the death phase. This experiment has been

repeated several times, and all of them showed the same growth pattern of *B. methanolicus*. The sudden increase in DO showed that *B. methanolicus* changed its growth behavior within two hours. In order to maintain healthy growth of *B. methanolicus*, the switch from batch to fed-batch or continuous operation should be made while *B. methanolicus* is in the exponential phase.

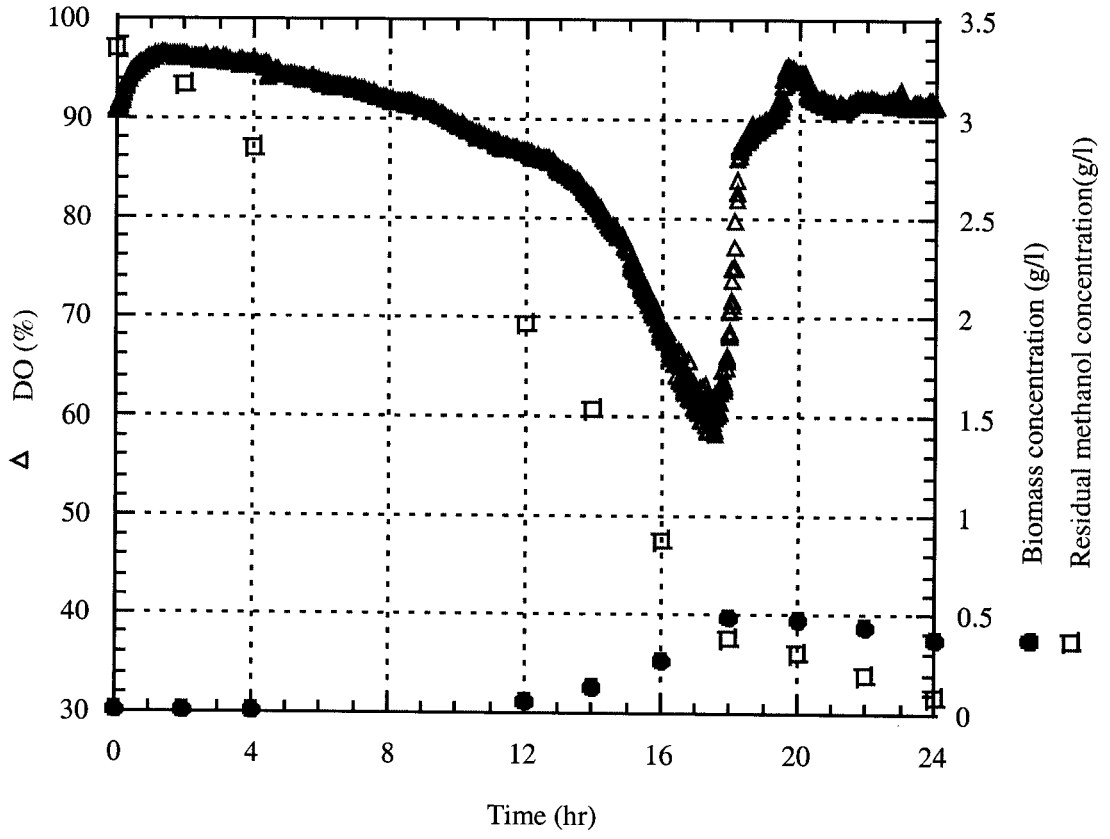

Figure 10. Growth of *B. methanolicus* in a 3-liter bioreactor under batch operation.

5.5.2 Batch/Fed-batch Operation

In order to obtain higher biomass concentration, *B. methanolicus* was grown under batch/fed-batch operation with initial methanol concentration 4.5 g/l. The system was switched from batch to fed-batch operation at t=22 hours. Optical density at 500 nm and methanol concentration were measured during the run. Figure 11 shows the growth of *B. methanolicus* under batch/fed-batch operation. Feed with 8 g/l methanol was added to the bioreactor with a feed rate of 100 ml/hr from t=22 to t=28 hours. Between t=36 to 40 hours, feed with 16 g/l methanol was added to the bioreactor with a feed rate of 100 ml/hr. To prevent the oxygen limitation, airflow rate and stirrer speed were changed to increase OTR. The operating conditions are shown in Figure 12. The specific growth rates and the methanol concentrations under different time periods are summarized in Table 9. As shown in Table 9, the specific growth rate decreased because of the sudden increase of methanol concentration.

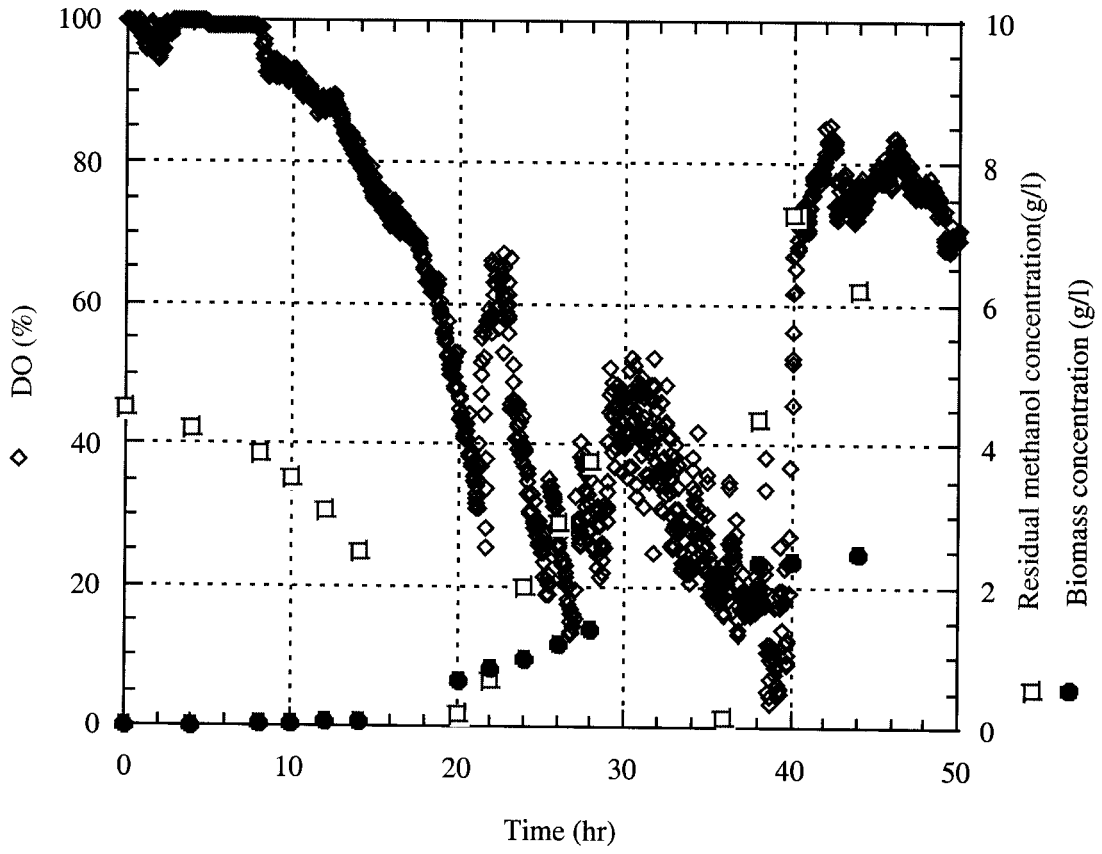

Figure 11. Growth of *B. methanolicus* in a 3-liter bioreactor under batch/fed-batch operation.

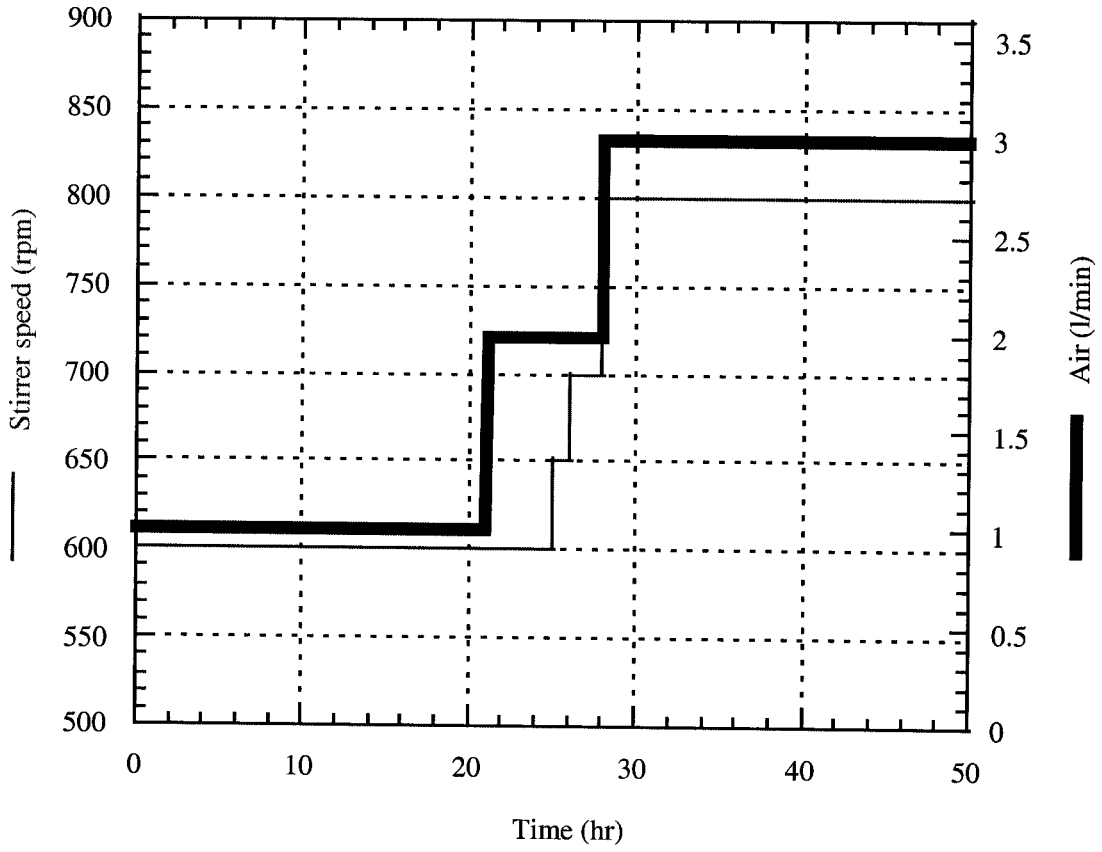

Figure 12. Operating conditions of stirrer speed and air flow rate vs. time for batch/fed-batch operation.

Table 9. Specific growth rate under different time periods.

Time (hr)	Specific growth rate (hr^{-1})	R^2
0~22	0.2839±0.0324	0.993
22~36	0.065±0.0040	0.987
36~40	0.0124±0.0036	0.854

5.5.3 Batch/Continuous Operation

The growth of *B. methanolicus* in a batch/continuous process is shown in Figure 13. The initial growth rate and Yx/s between 4 to 10 hours were calculated to be 0.34 hr^{-1} and 0.23 g/g. Figure 14 shows the data which were used to determine the initial growth rate. These results also show that the growth rate in the bioreactor at 0.34 hr^{-1} was higher than the growth in shake flasks at 0.251 hr^{-1}, and the Yx/s in bioreactor of 0.23 g/g was higher than for the growth in shake flasks with 0.128 g/g, as compared in Table 7 and 8. After 10 hours, different methanol feed rates were used during the run with the objective of maintaining a constant methanol concentration in the bioreactor. When the system reached a steady-state, air was shut off to determine the specific rate of oxygen consumption, q_{O2}.

As shown in Figure 15, the specific oxygen uptake rate decreased dramatically from 0.578 mg O_2/g cells-min at D=0.024 hr^{-1} to 0.326 mg O_2/g cells-min at D=0.072 hr^{-1}. However, the OUR decreased slightly from 0.3584 to 0.3392 mg O_2/l-min within the same range.

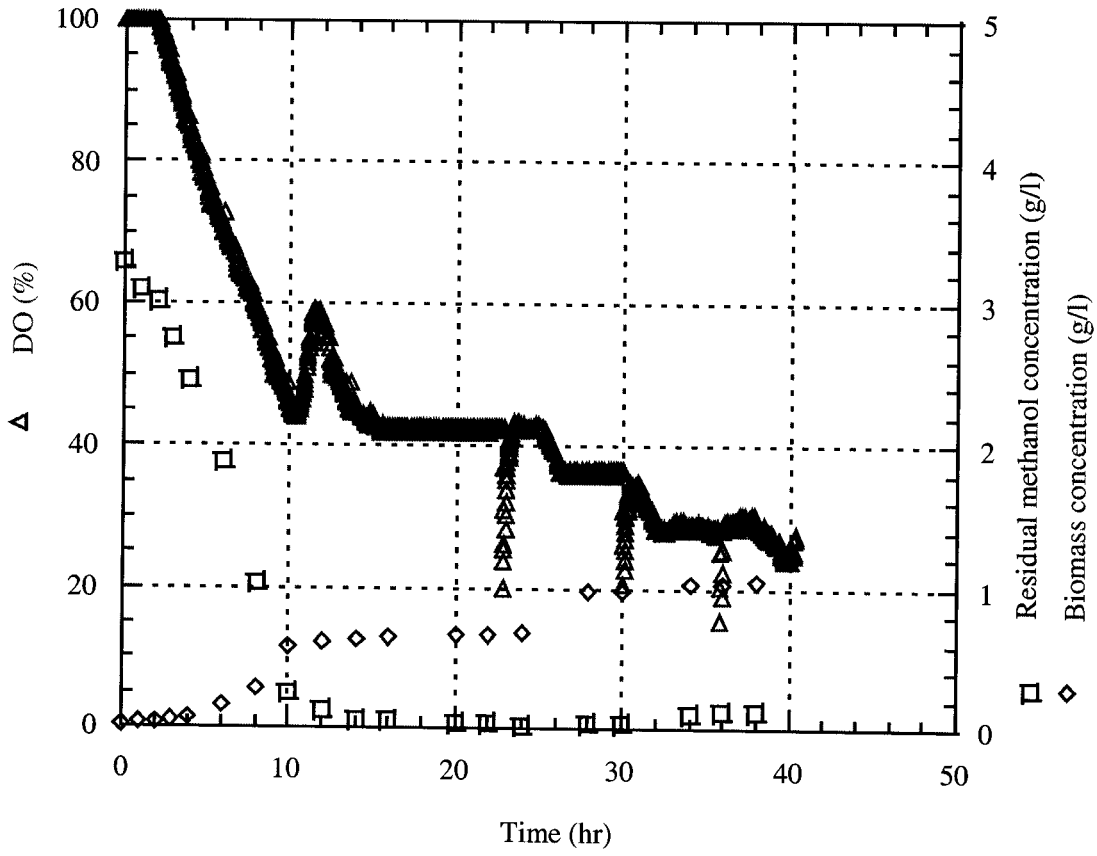

Figure 13. Growth of *B. methanolicus* in a 3-liter bioreactor under batch/continuous operation. The dilution rate between 10 to 22.5 hours, 22.5 to 31 hours, and 31 to 40 hours were 0.024, 0.06, and 0.072 hr^{-1}, respectively.

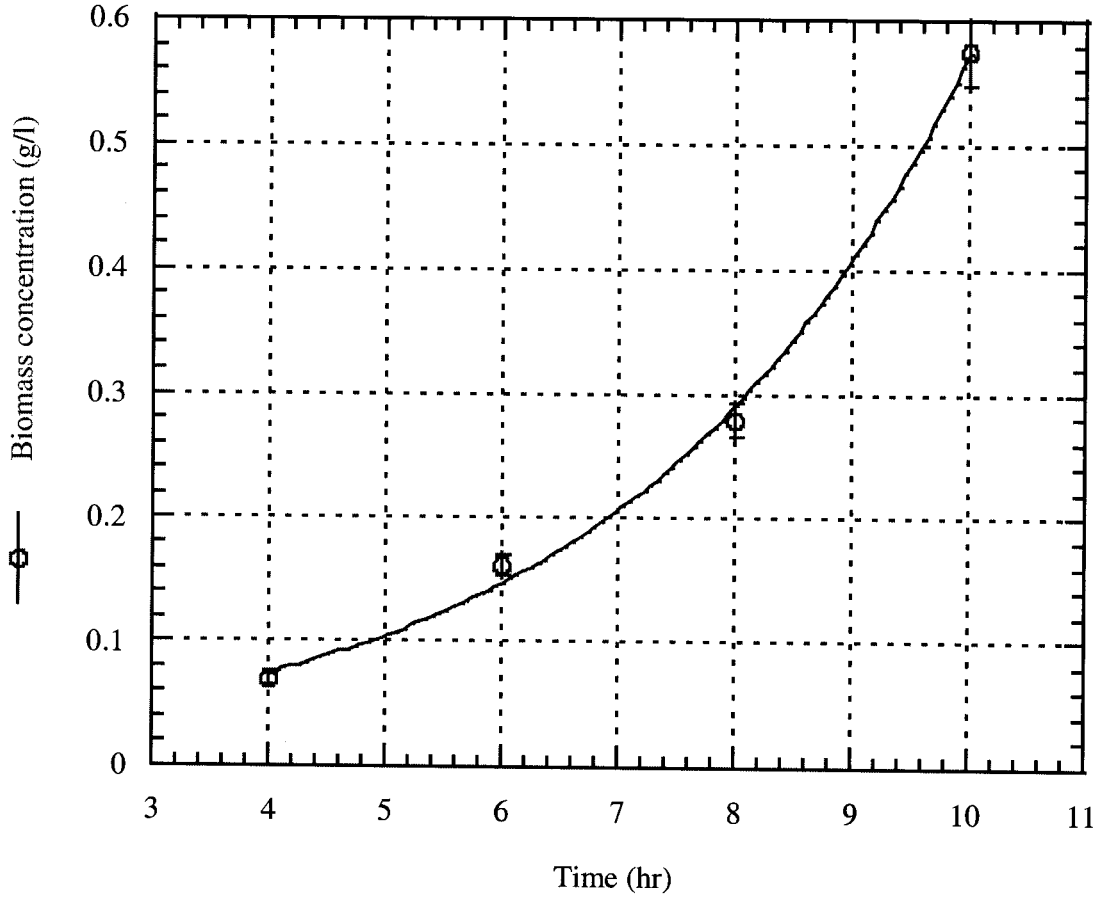

Figure 14. Initial growth rate of *B. methanolicus* in a 3-liter bioreactor. By the fitted curve, the specific growth rate was determined to be 0.341 hr^{-1}, R^2=0.997.

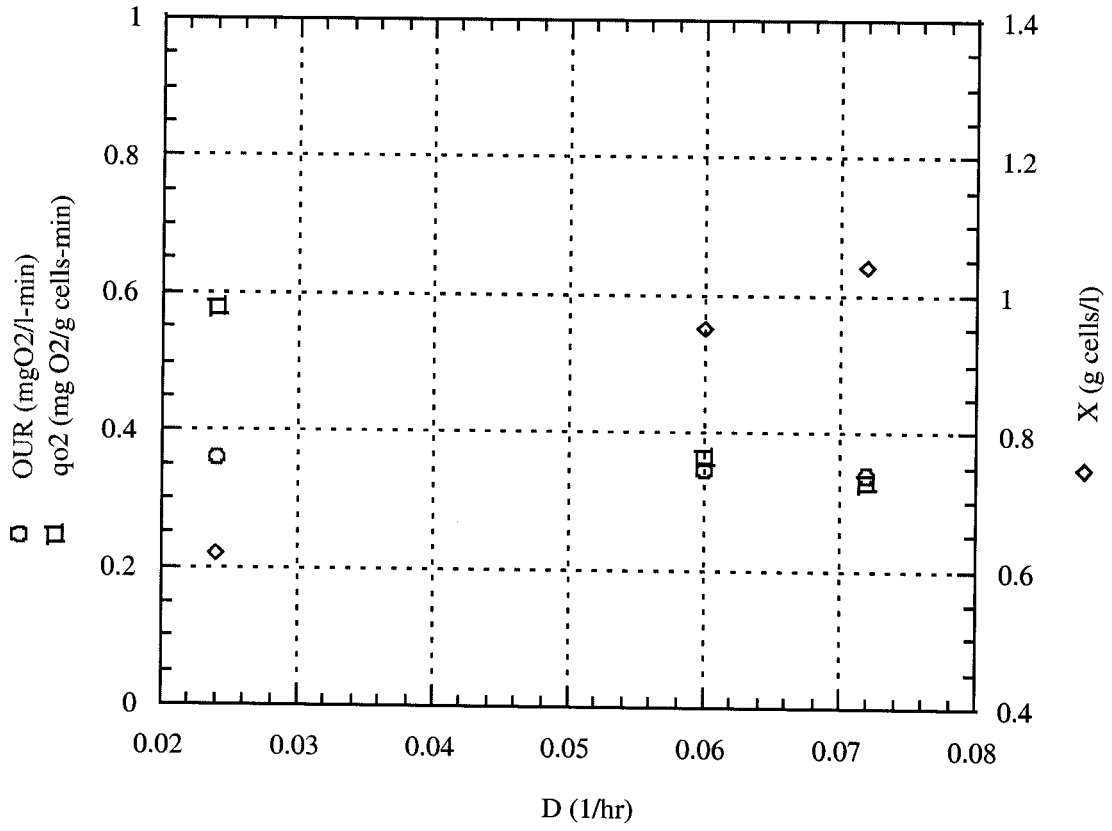

Figure 15. OUR, q_{O2} and X under different dilution rates.

5.5.4 The Biomass Yield on Methanol (Yx/s)

Six steady-state runs have been completed in a 3-liter bioreactor under continuous operation. Optical density and methanol concentration were measured during each run. Figure 16 shows that the dry biomass, residual methanol and Yx/s varied with dilution rate. The dry biomass concentration increased from 0.614 g/l at D=0.024 hr^{-1} to 1.096 g/l at D=0.12 hr^{-1}. The biomass yield showed a similar trend which increased from 0.194 to 0.366 g/g as the dilution rate was increased. $Y^{M}_{X/S}$, m_s and k_d have been determined to be the value of 0.46 g/g, 0.076 g methanol/g cells-hr and 0.0352 hr^{-1} by Figure 17, respectively. Pluschkell [3] reported the maximum yield of 0.5 grams of biomass per gram of methanol whereas the experiment conducted here shows a value of 0.46g/g.

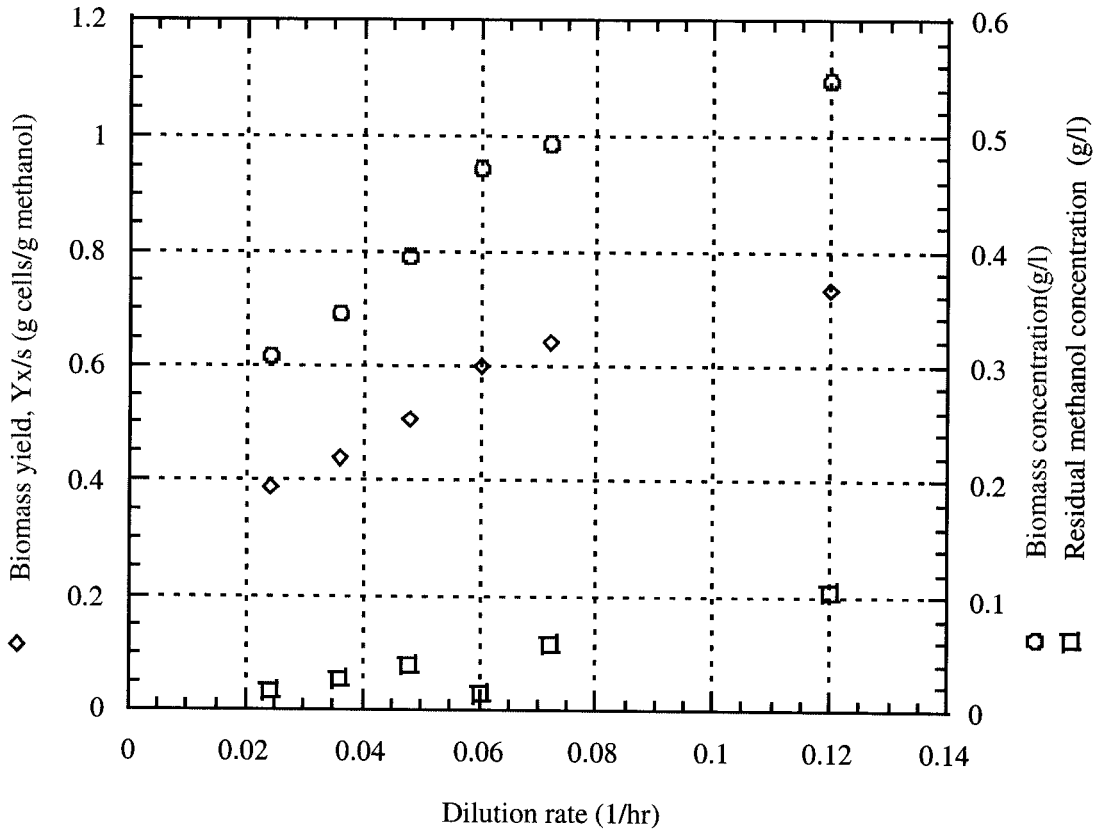

Figure 16. Continuous growth of *B. methanolicus* at steady-state as a function of the dilution rate.

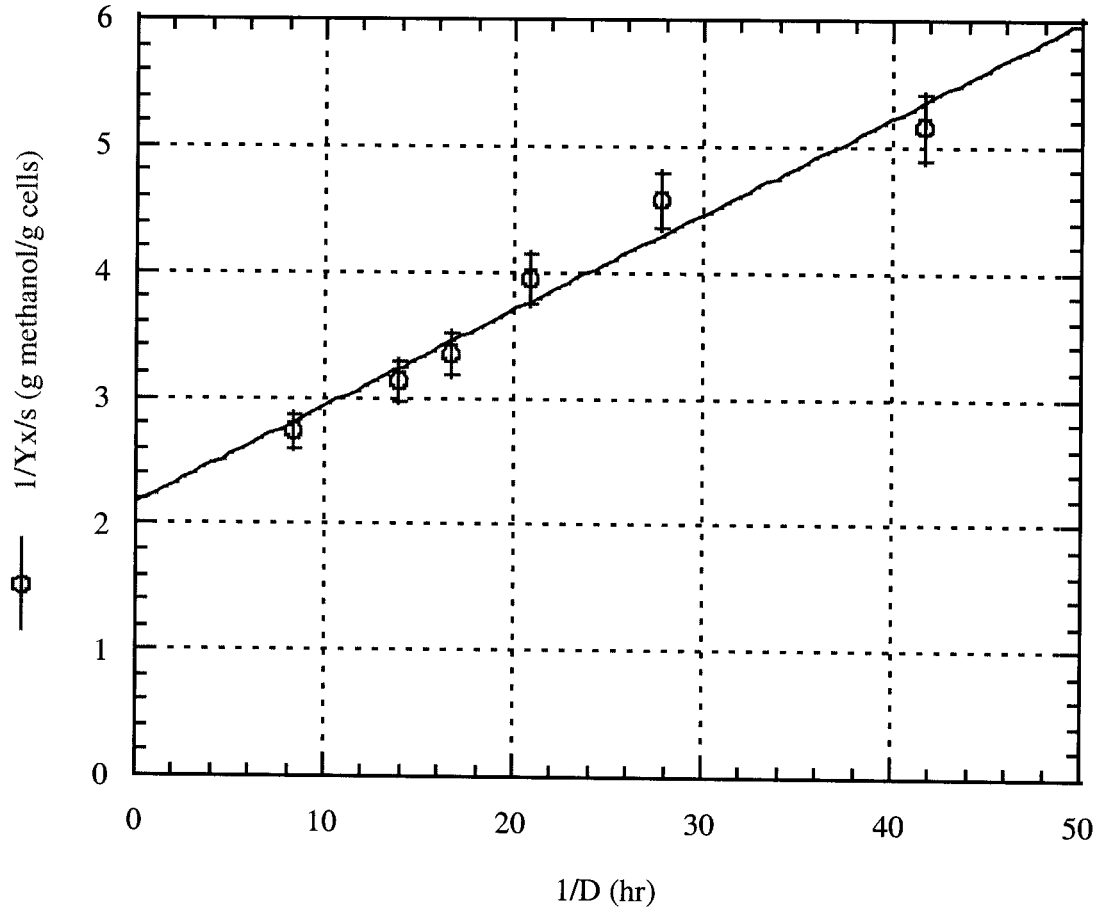

Figure 17. Graphical approach to estimate $Y^{M}_{X/S}$ and m_s for *B. methanolicus* growing on methanol as the limiting nutrient. By the regression line (R^2=0.959), the Y axis intercept which represents $1/Y^{M}_{X/S}$ is 2.171 g methanol/g cells, and the slope which represents m_s is 0.076 g methanol/g cells-hr.

5.5.5 Adaptive Control

The control of methanol concentration in the fermentation of *B. methanolicus* via adaptive control has been done under a batch/fed-batch operation. In this work, the control was "loose loop" control, whereby the operator was able to measure the methanol concentration offline and modify the feed rate based on the calculation with the adaptive control model.

The bioreactor was operated under batch condition for 8 hours. After 8 hours, the methanol concentration in the bioreactor was controlled via the adaptive control model under fed-batch operation. The initial conditions and set points for the adaptive control are shown in Table 10. Yx/s was estimated to be 0.39 g/g by Figure 17 at $D=\mu(0)=0.22$ hr^{-1}. In order to obtain the design parameters for the adaptive control model, the feed rate was controlled manually (not by the adaptive control model) for the first three hours, and the methanol concentration and optical density were measured. The values of three design parameters affected the estimated biomass and methanol concentration. As a result, the optimal values of design parameters were determined based on comparing the difference between measured and estimated data by changing different design parameter values. Figures 18 through 21 show the comparison between measured and estimated values for $\gamma_1=0.5$ and $\lambda_1=2$. On-line estimation of biomass, methanol concentration, and the specific growth rate were within 12.8%, 21.7%, and 21.1% of off-line measurements, respectively.

The performance of this adaptive control model can be improved by employing more correlations which are related to the time-varying parameters such as the specific growth rate and the biomass yield. Similar research for the fermentation of *Saccharomyces cerevisiae* indicated the specific growth rate and biomass yield were affected by the presence of product, ethanol [18]. By employing two correlations to modify the specific growth rate and biomass yield, a better performance of the same adaptive control model within 5% was showed by Thatipamala [18]. However, the growth kinetics of *B. methanolicus* is not clear at this point. The presence of product or substrate inhibition has not been analyzed. More researches on the growth kinetics of *B. methanolicus* need to be undertaken.

Possible errors in this work may be caused by measurement noise, sampling time, and fixed biomass yield. First, a ±10% error was observed for the UV/Visible spectrophotometer. For the determination of biomass concentration in the adaptive control experiment, samples were diluted up to twenty times. Therefore, the error may be amplified. Second, the sampling time can be shortened to thirty minutes or less instead of one hour which was used in this work. Finally, the correlations for biomass yield should be employed instead of using a fixed value because the performance of on-line estimation is seriously affected by the biomass yield.

Table 10. Initial conditions and setpoints for adaptive control.

$\hat{\mu}(0)$	0.22	hr^{-1}
$\hat{S}(0)$	0.54	g/l
Z(0)	0.93	g/l
X(0)	0.15	g/l
S(0)	0.54	g/l
D(0)	0.05	hr^{-1}
S^*	1.00	g/l
S_{in}	8.00	g/l
Yx/s	0.39	g cells/g methanol

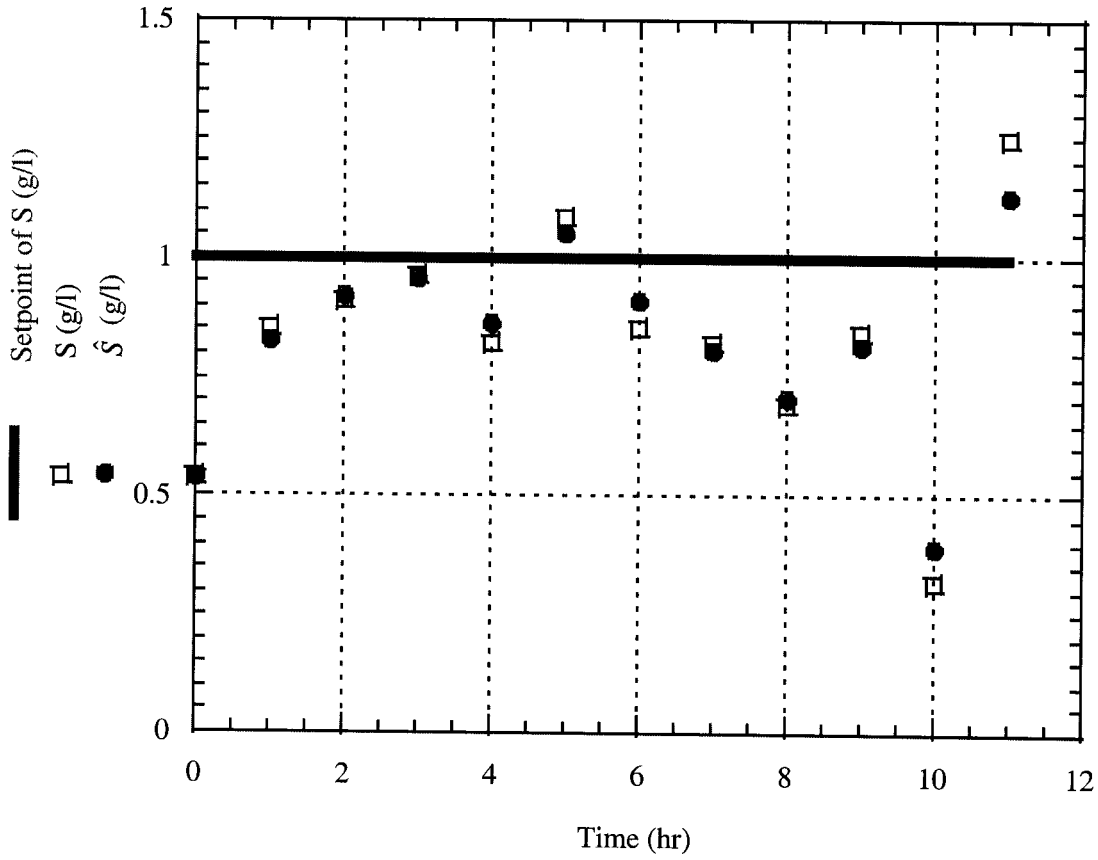

Figure 18. The comparison between measured (S) and estimated ($\hat{S}$) methanol concentration. The setpoint for methanol concentration was 1 (g/l).

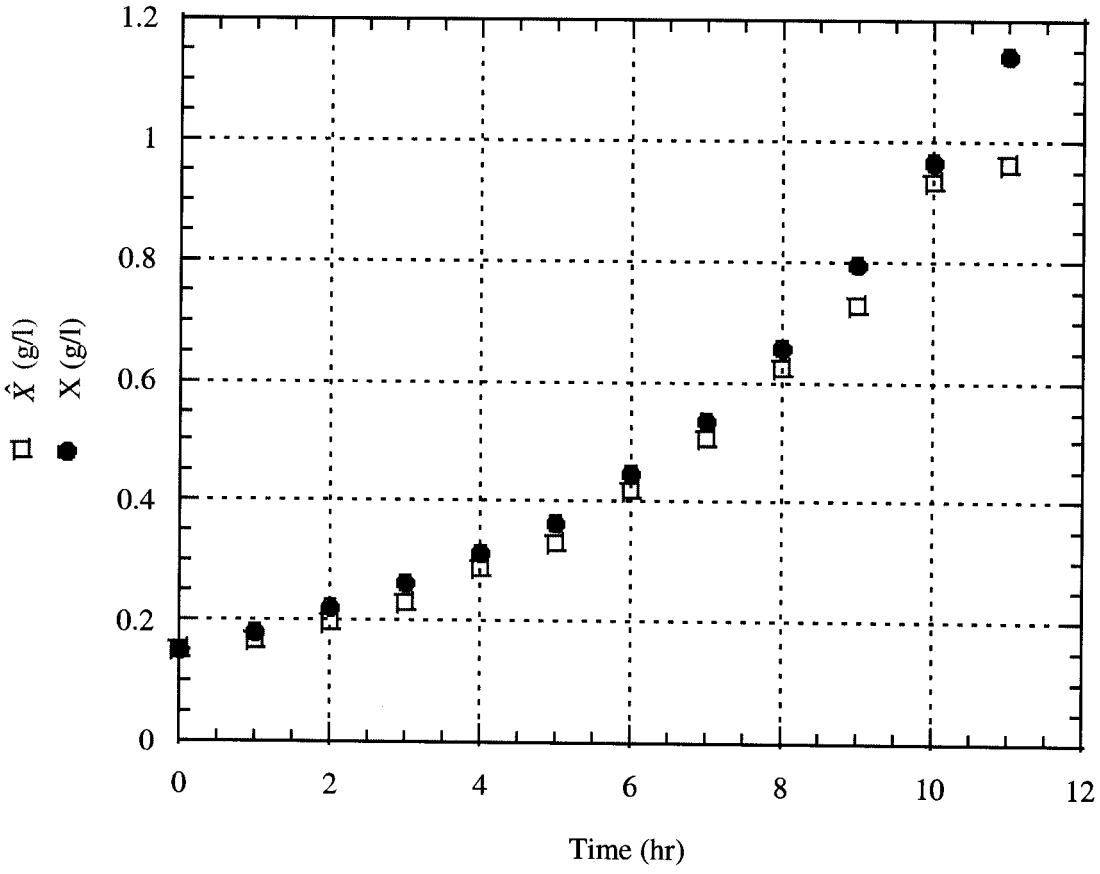

Figure 19. The comparison between measured (X) and estimated ($\hat{X}$) biomass concentration.

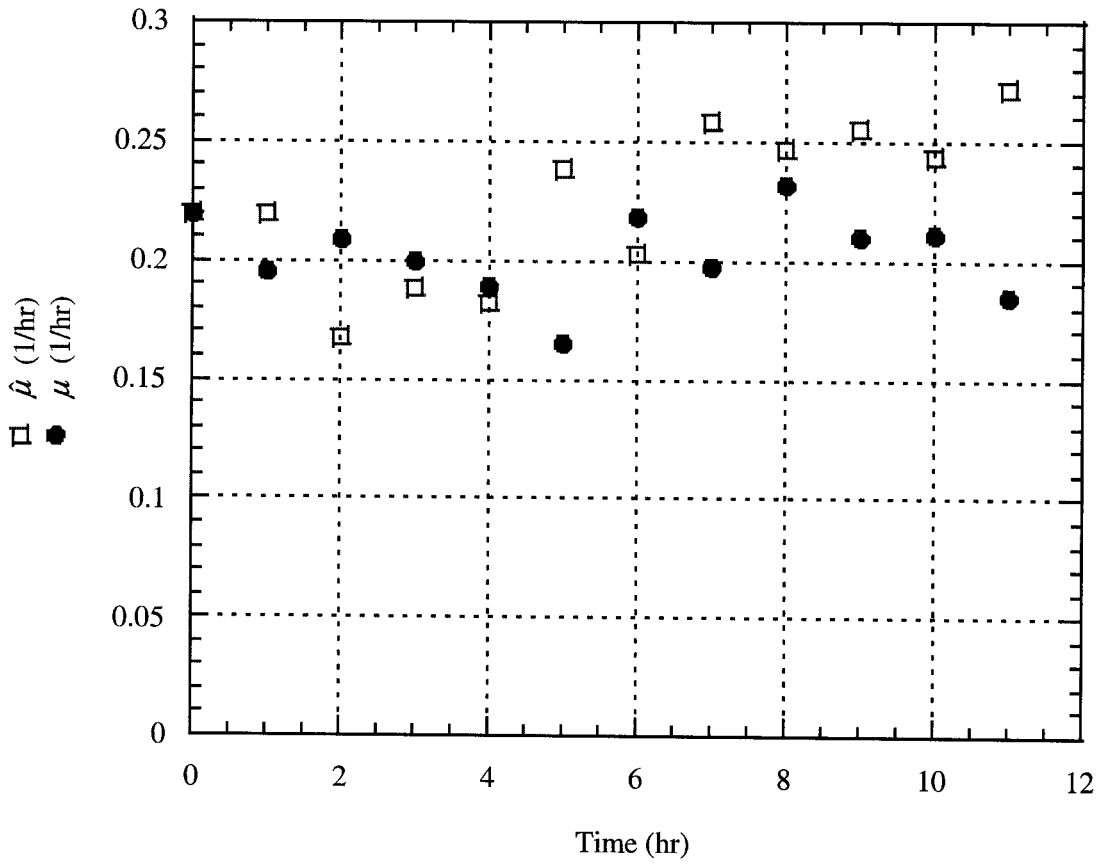

Figure 20. The comparison between measured (μ) and estimated ($\hat{\mu}$) growth rate.

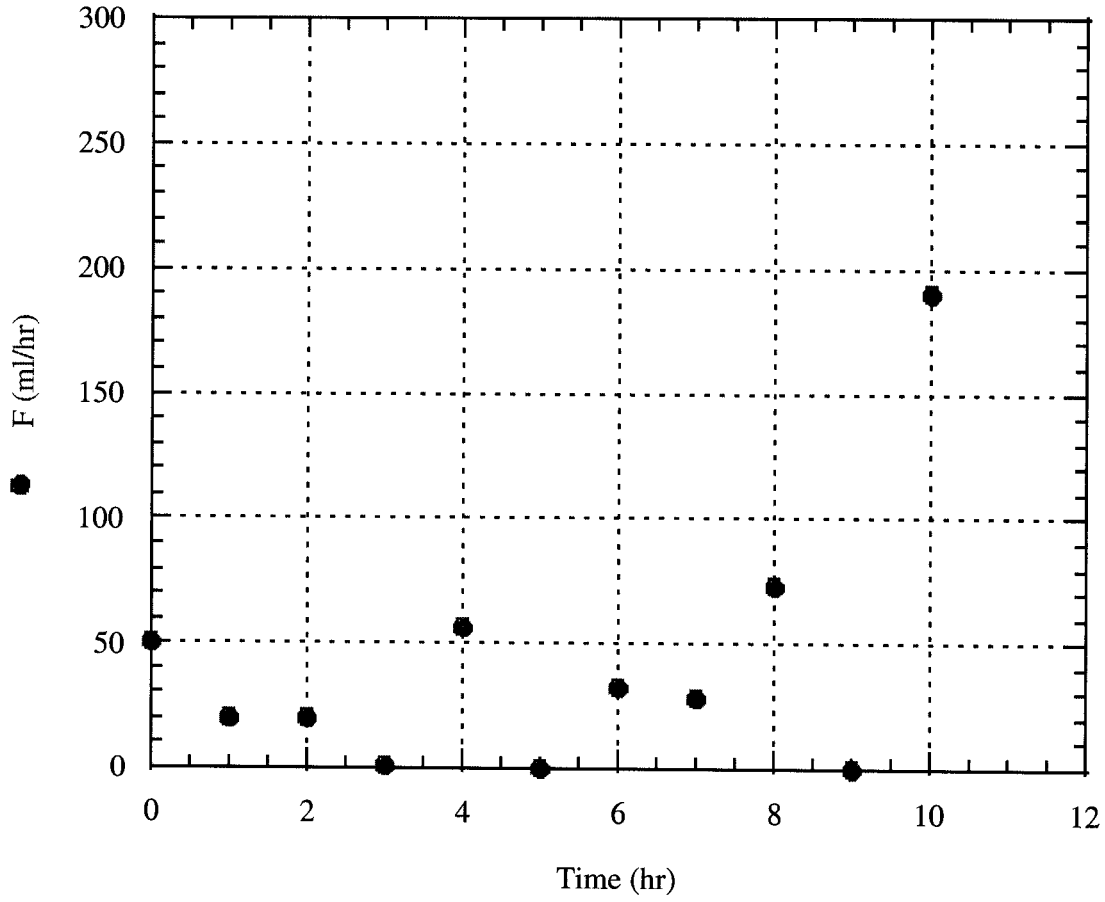

Figure 21. The feed rate versus time.

CHAPTER 6.0

CONCLUSIONS AND FUTURE RESEARCH

6.1 Conclusions

In order to control the methanol concentration in the fermentation of *B. methanolicus* via adaptive control, the yield of biomass on methanol, the specific growth rate, q_{O2}, and OUR have been determined under different operating conditions. The methanol concentration in the culture affects the growth behavior of *B. methanolicus*: the initial specific growth rate increased while the Yx/s decreased with increasing methanol concentration. Sudden changes of methanol concentration (methanol difference ≥ 1 g/l) also decreased the growth of *B. methanolicus*, and should be avoided. In addition, *B. methanolicus* can change it's growth behavior from exponential phase to stationary and dead phase within two hours which suggests that the switch from batch to fed-batch or continuous operation should be made while *B. methanolicus* is in the exponential phase. By knowing these possible restrictions, the control of *B. methanolicus* via adaptive control has been successively accomplished in this work. Only one process variable, the methanol concentration, is needed to be measured and is used to determine the feed flow rate. The specific growth rate and biomass concentration can be predicted via the adaptive control model within 21.1% and 12.8%, respectively.

6.2 Future Research

B. methanolicus is able to use methanol as its sole carbon and energy source. However, yeast extract, which contains some carbon sources, was used in the medium for both batch and continuous operations although it was not added in the feed for fed-batch and continuous operations. It could be an interesting topic for further researchers to investigate what ingredients in yeast extract are necessary for the growth of *B. methanolicus*.

Under continuous operation, *B. methanolicus* has been grown for more than 7 days with a biomass concentration 0.98 g/l. The maximum biomass concentration under fed-batch operation was 2.5 g/l (OD_{500}=17.5). However, the growth of *B. methanolicus* under fed-batch operation did not last for 2 days. It is possible that some toxic substances were produced and the concentration kept increasing during the fed-batch operation. Further research should focus on the cases of growth inhibition in cultures of *B. methanolicus*.

The carbon flux analysis could be another interesting topic. In order to approach this goal, researchers need to know the composition of biomass and the amount of glutamic acid and other possible byproducts which can be determined by a CHNS/O analyzer and LC/MS, respectively. In addition, the use of mass spectrometer to

determine the off-gas composition including oxygen, methanol, and carbon dioxide is also helpful. By knowing this information, the carbon flux model could be established and it would be helpful for the investigation of the metabolic pathways of *B. methanolicus.*

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